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TITLE OF THE INVENTION:

Mammalian subtilisin/kexin isozyme SKI-1: a proprotein convertase with a unique cleavage specificity

FIELD OF THE INVENTION:

This invention relates to a serine proteinase capable of converting proteic precursors into mature proteins; particularly a serine proteinase capable of cleaving at non-basic amino acid residues.

BACKGROUND OF THE INVENTION:

Limited proteolysis of inactive precursors to produce active peptides and proteins is an ancient mechanism to generate biologically diverse products from a finite set of genes. Most often, such processing occurs at either single or dibasic residues, as a result of cleavage by a family of mammalian serine proteinases related to bacterial subtilisin and yeast kexin(1, 2). These enzymes, known as pro-protein convertases (PCs), participate in the tissue-specific intracellular processing of precursors at the consensus **(R/K)**-$(X)_n$-**R**↓ sequence, where X is any amino acid except Cys and n = 0, 2, 4 or 6 (1-3). PCs have been implicated in the production of various bioactive polypeptide hormones, neuropeptides, enzymes, growth factors, adhesion molecules, cell surface receptors and surface glycoproteins of infectious agents such as viruses and bacteria (1-3).

Less commonly, bioactive products can also be produced by limited proteolysis at amino acids such as Leu, Val, Met, Ala, Thr, Ser and combinations thereof (3). This type of cellular processing has been implicated in the generation of bioactive peptides such as α-and γ-endorphin (4), the C-terminal glycopeptide fragment 1-19 of pro-vasopressin (5), anti-angiogenic polypeptides such as platelet factor 4 (6) and angiostatin (7), the metalloprotease ADAM-10 (8), site 1 cleavage of the sterol receptor element binding proteins (9), as well as in the production of the Alzheimer's amyloidogenic peptides Aβ40, 42 and 43 (10). Processing of this type occurs in the endoplasmic reticulum (ER) (9), or late along the secretory pathway, within secretory granules (4, 5), at the cell surface, or in endosomes (6-8, 10). So far, the proteinases responsible for these cleavages have not been unambiguously identified.

Since mammalian convertases process precursors at either single or pairs of basic residues, we hypothesised that a distinct, but related, enzyme(s) may generate

polypeptides by cleavage at non-basic residues. To test that idea, we employed an RT-PCR strategy similar to the one used to identify the PCs (11), except that we used degenerate oligonucleotides closer to bacterial subtilisin than to yeast kexin. This approach resulted in the isolation of a cDNA fragment encoding a putative subtilisin-like enzyme from human cell lines. This partial sequence was identical to a segment of a human myeloid cells-derived cDNA reported by Nagase *et al.* (12). A role for this putative subtilase remained undefined up to the present invention.

It was further discovered by Chang, D. *et al.* (1999) J. Biol. Chem. 274:22805-22812 that an enzyme called S1p, is capable of cleaving sterol-regulatory element-binding proteins (SREBPs), which function to control lipid biosynthesis and uptake in animal cells. Upon cleavage, SREBPs are released from cell membranes for translocation to the nucleus, where they activate transcription of genes involved in the biosynthesis and uptake of cholesterol and fatty acids. S1p and the present enzyme or the same. Therefore, for diseases involving overexpression of these genes as well as any other disease involving SKI-1 activity, it is contemplated that any inhibitor of SKI-1 would be useful in their treatment.

SUMMARY OF INVENTION:

We show that the sequences of the rat, mouse and human orthologues of this putative type-I membrane-bound subtilisin-kexin-isoenzyme, which we called SKI-1, exhibit a high degree of sequence conservation. Tissue distribution analysis by both Northern blots and *in situ* hybridization (ISH) revealed that SKI-1 mRNA is widely expressed. A stable transfectant of human SKI-1 in HK293 cells allowed the analysis of its biosynthesis and intracellular localization. We present data demonstrating that SKI-1 cleaves at a specific Thr↓ residue within the N-terminal segment of human pro-brain-derived neurotrophic factor (proBDNF). SKI-1 is the first identified secretory mammalian subtilisin/kexin-like enzyme capable of cleaving a proprotein at non-basic residues.

Therefore in accordance with the present invention, there is provided a soluble proteic fragment of a subtilisin-kexin isoenzyme named SKI-1 which has the amino acid sequence defined by amino acids 187 to 996 of any one SEQ ID NOs: 2, 4 and 6, a variant thereof, or an enzymatically active part thereof.

It is further an object of this invention to provide a proteic fragment of SKI-1 enzyme, which has the amino acid sequence defined by amino acids 18 to 137 of any one of SEQ ID NOs: 2, 4 and 6, a variant thereof, or a part thereof, which is a pro-segment capable of binding with amino acids 18 to 1052 of SKI-1 in whole or in part.

A part of this pro-segment has a molecular weight of about 14 KDa and forms a tight complex with the soluble fragment of SKI-1.

The pro-segment is an inhibitor of SKI-1 activity.

To improve its inhibitory activity, the pro-segment sequence is modified to prevent further enzymatic processing in a cell expressing said proteic fragment.

The modification includes amino acid substitution, deletion or rearrangement. Nucleic acids encoding any of the above SKI-1 forms are also objects of this invention.

Recombinant vectors and hosts comprising these nucleic acids are also objects of this invention.

The recombinant vectors are preferably expression vectors.

The recombinant vectors comprise a promoter expressible in a target cell wherein expression of said nucleic acid is desirable, be it for a therapeutic or manufacturing purposes.

The recombinant vectors may also comprise an inducible promoter.

It is further an object of this invention to provide a method of producing a proteic fragment of SKI-1 enzyme, which comprises the steps of:

culturing a recombinant host cell expressing a SKI-1 nucleic acid in a cell growth and expression-supportive culture medium; and recovering the proteic fragment of SKI-1 in the culture medium.

There is also provided a method for cleaving a proteic precursor which is an enzymatic substrate for SKI-1 enzyme, which comprises the step of:

a) contacting the proteic precursor with a SKI-1 enzyme which as an amino acid sequence defined by amino acids 18 to 1052 of SEQ ID Nos: 2, 4 or 6, or a variant thereof, or the soluble form, for a time sufficient and in condition adequate for such cleavage to occur.

The cleavage may be provoked *in vivo* or *in vitro*, e.g. serving a therapeutic purpose or an industrial protein manufacturing use.

For the purpose of producing a protein or a peptide from a proteic precursor which is an enzymatic substrate for SKI-1 enzyme, the method would further comprise the step of:

b) recovering and purifying the protein or peptide.

The method may be performed in cell-free assays, or may take place in a cell or in the presence of a cellular population, and wherein step a) comprises the step of transfecting a cell with a nucleic acid expressing a SKI-1 protein.

The cell may express said proteic precursor or may be transfected with a nucleic acid expressing the proteic precursor.

A method of silencing the expression or the activity of SKI-1 enzyme on a proteic precursor, which comprises the steps of:

contacting the enzyme or a nucleic acid encoding the enzyme with a ligand molecule which binds to the enzyme or to the nucleic acid, thereby interfering with the binding of the enzyme to the proteic precursor or with the expression of the nucleic acid encoding the enzyme, is also an object of this invention.

The ligand molecule may comprise an antisense nucleic acid to the nucleic acid encoding SKI-1, a pro-segment of a precursor protein encoding SKI-1, a SKI-inhibitor, a peptide mimicking a proteic precursor SKI-1 binding site, or an antibody molecule directed against SKI-1, or one which generates an inactive SKI-1 mutant form.

The pro-segment is a polypeptide extending from amino acids 17 to 137 of SEQ ID NOs: 2, 4, 6, or a variant thereof or an inhibitory part thereof.

We also provide a peptide of at least 7 amino acids capable of binding to and of being cleaved by SKI-1 catalytic active site, comprising the following general formula:

$$\text{Arg } Xaa_1 \text{ J } Xaa_2 \downarrow Xaa_3 (Z)_n \text{ O}$$

wherein $Xaa_{1,2,3}$ and Z are any amino acid

J is an alkyl or aromatic hydrophobic amino acid

n is 1, 2 or 3

O is an acidic amino acid.

Preferably Xaa_2 is Lys, Leu, Phe or Thr.

A preferred peptide has the structure:

H_2N-Val-Phe-Arg-Ser-Leu-Lys-Tyr-Ala-Glu-Ser-Asp-COOH.

The peptide may be labelled, a fluorogenic label being one of our preferred embodiments.

A fluorogenic peptide which has the following sequence:

Abz-Val-Phe-Arg-Ser-Leu-Lys-Tyr-Ala-Glu-Ser-Asp-Tyr(NO_2)

has been synthesized.

These peptides can be used for monitoring SKI-1 activity, for screening inhibitors of SKI-1 activity or for screening enhancers of SKI-1 activity.

An inhibitor of SKI-1 activity used in the making of a medication for treating a disease involving an overexpression of a SKI-1 or a SK1-1 substrate, is also a further object of this invention, namely the pro-segment modified or not.

The disease may be associated with any one of hypercholesterolemia, high levels of fatty acids, lipids or farnesyl pyrophosphate, liver steatosis, Ras-dependent cancer, restenosis and amyloid protein formation.

We also provide a method for detecting SKI-1 activity in a sample, which comprises the steps of contacting the sample with a ligand molecule to SKI-1 protein or nucleic acid, and detecting the formation of a complex between said ligand and SKI-protein or nucleic acid as an indication of the presence of SKI-1 in said sample. The ligand includes molecules such as anti-SKI-1-antibodies or a nucleic acid probes or primers.

Finally is provided a new use for SKI-1 enzyme in whole or in part which is for cleaving substrates not cleaved by other members of the subtilisin-kexin family. Variants of SKI-1 are under the scope of this invention, such variants are encoded by nucleic acids sharing at least 70% homology with the sequences defined in SEQ ID NOs: 1, 3, 5.

DESCRIPTION OF THE INVENTION:

During our search for new members of the subtilisin-kexin family, we obtained two closely related sequences from mouse and rat tissues. When questioning gene data banks to find a match with other known sequences, we found that the human counterpart has been previously cloned and sequenced. However, no specific function for this enzyme was known. We named our new enzyme subtilisin-kexin isoenzyme 1 (SKI-1).

We characterized this enzyme and found that SKI-1 has a unique cleavage site in cognate substrates. One of these substrates is pro-BDNF. Sakai *et al.* have found that another substrate, SREBP-2, which is a sterol-responsive transcription element, was cleaved at a first enzyme processing site by an enzyme which they called site 1 protease (S1p). S1p and SKI-1 appeared to be the same enzyme.

Since SKI-1 is autocatalytically cleaved, this brings to three the number of substrates that are known to be recognized and cleaved by SKI-1. One object of this invention is therefore the use of SKI-1 as a protein processing enzyme.

SKI-1 is ubiquitously distributed and appears to be very well conserved amongst mammalian species. Therefore, variants of SKI-1 are within the scope of this invention. We have indeed identified two species variants of the human enzyme disclosed in gene data banks, and *per se* this is a proof that variants to screen SKI-1 activity exist.

SKI-1 is first located in the endoplasmic reticulum (ER) membrane. Upon processing the pro-segment of pro-SKI-1 is removed and SKI-1 is thus activated. SKI-1 is further processed to remove the transmembrane domain that keeps it integrated in the ER membrane, which generates a SKI-1 soluble form that is directed into the secretory pathway and which remains active. The soluble active form is indeed retrievable in culture media as well as the pro-segment. The pro-segment is itself also processed into shorter fragments. One of these fragments has an apparent molecular weight of about 14 KDa and forms a tight complex with the soluble SKI-1 form. The formation of this complex does not hinder the activity of the enzyme. It is known that the pro-segment of pro-protein convertases is inhibitory *in vitro* to the activity of the convertases. We demonstrate for the first time hereinbelow that such a behaviour occurs in an *ex vivo* model. SKI-1 pro-segment also has such an inhibitory activity. We predict that a SKI-1 pro-segment that would be modified to prevent the pro-segment processing will be an even better SKI-1 inhibitor. Such a modification is made by converting an enzyme recognition and cleavage site into a non-cleavable sequence. Such modification is intended to cover amino acid substitutions, deletions or re-arrangements to provide a SKI-1 pro-fragment that has an improved inhibitory activity.

The nucleic acids encoding all the above SKI-1 forms (soluble, pro-segment and sub-fragments, modified or not) are under the scope of this invention. Recombinant vectors and hosts comprising these nucleic acids are also objects of this invention. More particularly, expression vectors capable of producing the different SKI-1 forms are preferred. The expression vectors comprise promoter sequences which govern the expression of the nucleic acids. The promoter may be compatible with the cell wherein the expression of the nucleic acid is sought, be it for a therapeutic purpose or for the industrial production of SKI-1. The promoter may also be an inducible promoter which needs an exogenous inducing agent to activate the expression. For

the production of any SKI-1 form, a recombinant host cell may be used and is cultured in a culture medium which supports cell proliferation and the expression of the nucleic acids. Under suitable conditions, the SKI-1 form of interest is expressed and may be conveniently recovered from the culture medium.

A general method for cleaving a proteic precursor is also an object of this invention. SKI-1 whole active enzyme or its soluble form or catalytically active fragments or variants are added to a proteic precursor which is a SKI-1 substrate, in conditions adequate for enzymatic precursor processing (cleavage) to occur. This method may be performed *in vivo* for curing a SKI-1 deficiency or *in vitro* for the industrial preparation of active proteins. In the latter case, the processing may be performed in a cell-free assay, using purified proteic precursors and SKI-1 whole enzyme or derived forms. Alternatively, it may be performed using transfected cells expressing SKI-1 whole enzyme and derived forms. The transfected cells may endogenously express the protein precursor or may be co-transfected to express the same. The transformed cells therefore become a manufacture of mature proteins and/or or SKI-1.

Modification of the SKI-1 activity is further an object of this invention. We have succeeded in inhibiting SKI-1 activity using the SKI-1 pro-segment. Alternative ways to achieve the same results include antisense nucleic acids or oligonucleotides, SKI-1 inhibitors, peptides mimicking a precursor SKI-1 binding site (cleavable or not), which would compete for the binding of SKI-1 to its cognate protein precursor site, and antibodies directed against SKI-1 or its cognate proteic precursor binding site. Another alternative is a genic therapy replacing the active SKI-1 by an inactive mutant form. On the opposite, overexpressing SKI-1 may cure a SKI-1 deficiency. Due to the ubiquitous distribution of SKI-1, it may be useful, even necessary, to target the cell wherein SKI-1 activity is to be modified for such a therapeutic purpose. Such targeting may include conjugating or combining molecules capable of modifying or modulating SKI-1 activity to a ligand capable of targeting the cell of interest. Immunoliposomes are examples of targeting vehicles as well as conjugated ligands-oligonucleotides. Even viral vectors may be made targeting if they express such a targeting ligand at the membrane surface. A targetting ligand serves a selection purpose, leaving substantially intact the non-targetted cells.

Peptides of less than 100 amino acids, more preferably of less than 30 amino acids, mimicking a cognate SKI-1 cleaving site in a proteic precursor have been synthesized and are also objects of this invention. Therefore, a peptide of at least 7 amino acids comprising the following preferred structure is capable of binding to and of being cleaved by SKI-1 enzyme catalytic site:

$$\text{Arg } Xaa_1 \text{ J } Xaa_2 \downarrow Xaa_3 (Z)_n \text{ O}$$

wherein $Xaa_{1, 2, 3}$ and Z are any amino acid

J is an alkyl or aromatic hydrophobic amino acid

n is 1, 2 or 3

O is an acidic amino acid.

Preferably Xaa_2 is Lys, Leu, Phe or Thr.

The preferred peptide has the following sequence:

H_2N-Val-Phe-Arg-Ser-Leu-Lys ↑ Tyr-Ala-Glu-Ser-Asp-COOH.

These peptides may be labelled in such a way that labelled fragments produced upon cleavage are easily detected and identified. Such labelling include any type of suitable detectable markers. We have developed a fluorogenic peptide which shows a very good affinity for SKI-1. The above preferred peptide has been labelled at its N- and C- terminal ends with an orthoaminobenzoic acid and 3-nitrotyrosine groups, respectively.

These peptides as well as cell lines expressing SKI-1 will be especially useful for monitoring SKI-1 activity and for screening inhibitors or substrates and enhancers of SKI-1 activity.

Inhibitors of SKI-1, namely the SKI-1 pro-segment, will be used in the making of a medication for treating a diseasing involving overexpression of SKI-1 or of its substrate.

Conversely, substrates of SKI-1 will be used in the research field to discover physiological systems involving SKI-1.

Diagnostic methods and kits comprising a ligand to SKI-1 protein or nucleic acid, which is to be contacted with a sample suspected to express SKI-1, is also an object of this invention. Detection of the formation of a ligand-SKI-1 complex or of a hybridization complex is an indication of the presence or amount of SKI-1 in the sample.

Since we were the first to discover the function of SKI-1 enzyme, the use thereof for cleaving proteic precursors that are not substrates for the other members

of the subtilisin-kexin family is an object of this invention. SKI-1 is intended in this broad use to cover the whole enzyme, a catalytic part thereof and its functional variants. Variants are encoded by anyone of the nucleic acids depicted in SEQ ID Nos: 1, 3 or 5, and any other sequences sharing at least 70% homology therewith, preferably more than 85% homology, under stringent conditions of hybridization.

Having now defined the general teachings of the present invention, reference will be made hereinbelow to specific examples and embodiments as well to the following appended figures, which purpose is to illustrate the invention rather than to limit its scope.

BRIEF DESCRIPTION OF FIGURES:

FIG. 1 shows the comparative protein sequences of SKI-1 deduced from rat, mouse and human cDNAs (SEQ ID NOs 2, 4, and 6, encoded by nucleic acids SEQ ID NOs: 1, 3, and 5, respectively). The position of the predicted end of the 17 aa signal peptide is shown by an arrow. The active sites Asp^{218}, His^{249} and Ser^{414}, as well as the oxyanion hole Asn^{338} are in bold, shaded and underlined characters. The positions of the 6 potential N-glycosylation sites are emphasized in bold. The conserved shaded **CL**DDSHRQK**DC**F**W** sequence fits the consensus signature for growth factors and cytokine receptors family. Each of the two boxed sequences was absent in a number of rat clones. The predicted transmembrane segment is in bold and underlined.

FIG. 2 shows a Northern blot analysis of the expression of SKI-1 in adult rat tissues. **[A]** 5 µg of male rat total RNA were loaded in each lane. Molecular sizes are based on the migration of an RNA ladder. The tissues include: adrenal, thyroid, striatum, hippocampus, hypothalamus, pineal gland, anterior (AP) and neurointermediate (NIL) lobes of the pituitary, submaxillary gland, prostate, ovary and uterus. Notice the high level of SKI-1 mRNA in adrenal glands. **[B]** 2 µg of poly-A+ of (male + female) Sprague Dawley rat adult tissues (Bio/Can Scientific) were loaded, which includes: liver, thymus, spleen, kidney, heart and brain. The estimated size of rat SKI-1 mRNA is about 3.9 kb.

FIG. 3 shows *in situ* hybridization (15 H) of rSKI-1 mRNA in a 2 day old rat. ISH is shown at anatomical resolution on X-ray film using an [^{35}S]-labeled antisense riboprobe **[A-C]** and sense control riboprobe **[D]**. Abbreviations: *Adr* - adrenal gland; *Cb* - cerebellum; *cc* - corpus callosum; *Cx* - cerebral cortex; *H* - heart; *Int* - intestine; *K* - kidney; *Li* - liver; *Lu* - lungs; *M* - muscles; *Mol* - molars; *OT* - olfactory turbinates; *Pit* - pituitary gland; *Rb* - ribs; *Ret* - retina; *Sk* - skin, *SM* - submaxillary gland; *Th* - thymus. Magnification x 4; scale bar (in **D**) = 1cm.

FIG. 4 illustrates the biosynthetic analysis of SKI-1 in HK293 cells. Stable transfectants expressing either the pcDNA3 vector alone or one that expresses SKI-1 (clone 9) were pulse-labeled for 4h with [^{35}S]Met. Media and cell lysates were immunoprecipitated with either a SKI-1 antiserum (Ab: SKI; against aa 634-651) or a pro-SKI-1 antiserum (Pro). The stars represent the 4 specific intracellular proteins (Mr 148, 120, 106 and 98 kDa) immunoprecipitated with the SKI-1 antiserum. In these transfected cells, only the 148 kDa band is recognized by the Pro-antiserum. A 98 kDa immunoreactive SKI-1s protein is also detectable in the medium.

FIG. 5 shows hSKI-1 immunoreactivity in stably transfected HK293 cells. Representation of the comparative double fluorescence staining using a SKI-1 antiserum (directed against aa 634-651) [A] and [B] and FITC-labeled WGA [A'] and [B'] in control [A, A'] and LME-treated [B, B'] cells is shown. Thin arrows emphasize the observed punctate staining which is enhanced in the presence of LME. Large arrows point to the coincident staining of SKI-1 and WGA. Magnification x 900; bar (in B') = 10 µm.

FIG. 6 shows the processing of proBDNF by SKI-1. **[A]** COS-7 cells were infected with vv:BDNF and either vv:WT (-) or vv:SKI-1 in the presence of either vv: PIT or vv:PDX. The cells were metabolically labeled with [^{35}S]Cys-Met for 4h and the media (M) and cell lysates (C) were immunoprecipitated with a BDNF antiserum, prior to SDS-PAGE analysis. The autoradiogram shows the migration positions of proBDNF (32 kDa), the 28 kDa BDNF produced by SKI-1 and the 14 kDa BDNF. **[B]** Microsequence analysis of the [^{35}S]Met-labeled 32 kDa proBDNF (maximal scale 1000 cpm) and [^{3}H]Leu-labeled 28 kDa BDNF (maximal scale 250 cpm), revealing a Met at sequence position 3 and Leu at positions 2, 13 and 14, respectively.

FIG. 7 shows the *in vitro* processing profile of proBDNF by SKI-1. **[A]** pH dependence of the processing of proBDNF by SKI-1. The SKI-1 enzyme preparation was compared to that obtained from the media of Schwann cells infected with the wild type virus (WT) as control. **[B]** Inhibitor profile of the processing of proBDNF to the 28 kDa BDNF by the same SKI-1 preparation as in **[A]**. The reaction was performed overnight at 37°C, pH 6.0. Notice that only PMSF (0.5 mM PMSF+50 µM pAPMSF), o-phenanthroline (5 mM), and EDTA (10 mM) effectively inhibited SKI-1 cleavage of proBDNF.

FIG. 8 shows the *in situ* hybridization translating SKI-1 mRNA expression in the pituitary gland of an adult rat using specific [^{35}S]radiolabeled antisense (*SKI AS*) and control sense (*SKI SS*) riboprobes. The hybridization signal was detected in the anterior (AL), intermediate (IL) and posterior pituitary lobe (PL). Most of the labeling was confined to endocrine cells in AL and IL and to some pituicytes in the PL. Magnification x 5; bar (in b) = 1 mm.

FIG. 9 shows the *in situ* hybridization translating the presence of SKI-1 mRNA sites in the skin of a newborn two days old (p2) rat using antisense (*SKI AS*) and control sense (*SKI SS*) riboprobes. The hybridization signal was detected in the stratum germinativum (small vertical arrows in SGe), in both outer and inner hair sheath (medium arrows) and in some cells within the dermis (D). Other abbreviations: HB - hair bulb, SC - stratum corneum, SGr - stratum granulosum. Magnification x 80.

Fig. 10 shows the *in situ* hybridization (ISH) distribution of SKI-1 mRNA in the rat central nervous system (CNS). ISH distribution pattern in the CNS of adult rat demonstrates a higher concentration of SKI-1 mRNA within a grey matter (GM and all structures indicated with capital letters) vs the white matter (WM) including corpus callosum (cc). Representative brain structures are shown in sagittal (a); horizontal (b) and coronal plane (c - f) after hybridization with antisense SKI-1 riboprobe (a - e) and control sense riboprobe (ssRNA in f). As shown at anatomical level this type of mRNA distribution is highly reminiscent to a type of pan-neuronal gene distribution pattern. As complementary to this figure a Table 1 demonstrates at cellular level the predominance of neuronal SKI-1 mRNA expression over glial SKI-1 mRNA expression. Magnification x 4; bar (in a) = 1 cm. Abbreviations: **CA1** - area 1 of cornus Ammonis; **CA3** - area 3 of cornus Ammonis; **Cb** - cerebellum; **cc** - corpus callosum; **Ch Pl** - choroid plexus; **Cx** - cerebral cortex; **GD** - gyrus dentatus; **GM** - grey matter; **Hip** - hippocamp; **Hy** - hypothalamus; **Ol** - olfactory bulb; **Str** - striatum; **WM** - white matter.

Fig. 11 shows the *in situ* hybridization (ISH) distribution of SKI-1 mRNA in the rat peripheral nervous system (PNS) trigeminal ganglion (TriG). ISH distribution pattern in the CNS of adult rat demonstrates a higher concentration of SKI-1 mRNA within a region of cell bodies (large arrows) over the region of supportive Schwann cells (small arrows). ISH was performed using antisense (SKI-1 as in a) and sense (SKI-1 ss) riboprobes. Magnification x 12.

Fig. 12 shows the distribution of SKI-1, mRNA and/or protein, in the region of spinal cord (SpC) and in the related dorsal root ganglion (DRG) and dorsal root (DR). Demonstrated are the region of neuronal cell bodies in the DRG (SKI-1 mRNA) and the region of nerve terminals in the dorsal horn of the spinal cord (layer I and II) characterized by a especial density of SKI-1 protein.

A) Schematic drawing depicting the position of layer I and II in the dorsal horn as well as that of the related DRG and DR.

B) SKI-1 mRNA revealed by *in situ* hybridization labeling (thin arrows) in the DRG using antisense riboprobes (*SKI-1 AS*).

C) Control hybridization in the DRG using sense riboprobes (*SKI-1 SS*).

D) Immunocytochemical localization of SKI-1 (brown staining) within layer I and II of the dorsal horn and in the dorsal root (DR) suggesting the sensory afferents arriving from DRG. Neuronal and glial nuclei are stained on blue. Magnification x 300.

E) Immunoreactivity of SKI-1 (thin arrows) detected around neuronal somata (large arrows) within layer II of the dorsal horn at high magnification (x 1,500). Pattern of immunoreactive spots is reminiscent to that of axo-somatic or axo-dendritic nerve terminals.

F) Northern blot revealing the concentrations of 4 kb SKI-1 mRNA in different tissues including dorsal root ganglia (DRG) and spinal cord (SpC). Abbreviations: **I** - layer I of the dorsal horn; **II** - layer II of the dorsal horn; **Adr** - adrenal gland; **Cb** - cerebellum; **Cx** - cerebral cortex; **Hip** - hippocamp; **DH** - dorsal horn; **DR** - dorsal root; **DRG** - dorsal root ganglion; **SpC** - spinal cord; **Stom** - stomach and **Thyr** - thyroid gland.

Fig. 13 shows the farnesyl diphosphatase mRNA levels in HK 293 cells treated with (+)lipids (cholesterol and 25-hydroxycholesterol) or without lipids (-). 1-2 = wild type cells, 3-4 = SREBP-1 overexpressors, 5-6 = a pool of 3 different clones overexpressing SREBP-1 and Pro-SKI-1 ; clones 4,6,9.

Fig. 14 shows the fatty acid synthase mRNA levels in HK 293 cells treated with (+) lipids (cholesterol and 25-hydroxycholesterol) or without lipids (-). 1-2 = wildtype cells, 3-4 = SREBP-1 overexpressors, 5-6 = a pool of 3 different clones overexpressing SREBP-1 and Pro-SKI-1; clones 4,6,9.

Fig. 15 shows the HMG CoA reductase mRNA levels in HK 293 cells treated with lipids (box A) or without lipids (box B). 1 = wild type cells, 2 = vector only cells, 3 = SREBP-1 overexpressor cells, 4 = SREBP-1 and ProSKI-1 overexpressor cells (high SREBP expression, clone 6), 5 = SREBP-1 and ProSKI-1 overexpressor cells (low SREBP expression, clone 9).

Fig. 16 shows the HMG CoA reductase and farnesyl diphosphatase mRNA levels in Hk 293 cells in different clones overexpressing SREBP-1 (1-5) or SREBP-1 and ProSki-1 (clone 4, clone 6, clone 9). Cells were treated with fetal calf serum.

Fig. 17 shows the nuclear SREBP-1 in HK 293 cells in absence of lipids. Mature SREBP is processed in the ER and translocated into the nucleus. 1 = wild type cells, 2 = vector only cells, 3 = SREBP-1 overexpressors, 4 = SKI-1 antisense cells, 5 = ProSki + SREBP-1overexpressors clone 6, 6 = ProSKI + SREBP-1 overexpressors clone 9.

Fig. 18 shows the processing of cytoplasmic SREBP-1 in HK 293 cells. 50 µg of protein per lane was separated in 6 % (above) and 10 % (below) SDS-PAGE gels. Uncut SREBP-1 (proSREBP-1) and intermediate SREBP-1 (intSREBP-1) cleaved by SKI-1 are indicated with arrows. Cell lines express ProSKI-1 (pSKI), SKI-1 anti-sense (SKI-1 as), SREBP-1, or ProSKI-1 and SREBP-1 (pSKI + SRE), or control vector (pcDNA3), as indicated. Analysis was performed in the presence (+ sterols) or absence of sterols (- sterols).

Fig. 19 [A] is a schematic representation of the structure of FL-SKI-1 and its truncation mutant BTMD-SKI-1. The various SKI-1 domains depicted are, respectively, the signal peptide, pro-segment, catalytic domain, and the C-terminal region comprising a cytokine receptor/growth factor motif, a transmembrane domain and a cytosolic tail. The positions of polypeptides used to produce SKI-1-specific antisera (Ab: *P*, *N* and *S*) are also displayed. Fig. 19 [B] shows the biosynthetic analysis of SKI-1. VV:FL-SKI-1, BTMD-SKI-1 (bSKI-1) or control VV:WT infected LoVo cells were pulse-labeled with [^{35}S]Cys for 3h. Media were immunoprecipitated with either Ab:*S* or Ab:*P* and then resolved by SDS-PAGE on an 8 % gel followed by autoradiography. Arrows point to the migration positions of the 100 kDa BTMD-SKI-1 (bSKI-1), the 98 kDa shed form

(sSKI-1) as well as the 14 kDa prosegment product. Fig. 19 [C] shows a Western blot analysis of the overexpressed BTMD-SKI-1. Samples from VV:WT or BTMD-SKI-1 infected BSC 40 cells (left and middle panel) were processed as described in "Experimental Procedures" and run on an 8 % SDS-PAGE reducing gel. Following electrotransfer to PVDF membranes, protein bands were visualized via ECL detection using primary rabbit antisera Ab:*S* or Ab:*N*. Purified BTMD-SKI-1 (right panel, *) was obtained from a Ni^{2+} affinity resin as described in "Experimental Procedures", then processed as described above. A mixture of Ab:*S* and Ab:*P* were used as primary antisera. Elution buffer was used as a control (CTL).

Fig. 20 shows the biosynthetic analysis of the rate of zymogen processing and the fate of the prosegment of SKI-1. LoVo cells overexpressing VV:FL-SKI-1 were pulse-labeled with [^{3}H]Leu for 15 min and then chased for 2h (P15C2h), or pulsed for 2h in the presence or absence of BFA (P2h). Cell lysates were immunoprecipitated with Ab:*P*, resolved by SDS-PAGE on a 14 % gel and autoradiographed. The migration positions of the major ~26, 24, 14, 10 and 8 kDa prosegments are emphasized.

Fig. 21 illustrates the purification and identification of secreted recombinant pro-SKI-1. [A] Media obtained from HK293 cells stably expressing FL-SKI-1 were concentrated and sequentially applied to C4 semi-preparative column (*not shown*) followed by a C4 analytical RP-HPLC columns, and then eluted by the indicated linear CH_3CN gradient. **[B]** The fractions labeled I-IV were collected and analyzed by Western blotting using the primary antiserum Ab:*P*. **[C,D]** Proteins contained in fraction IV were separated on a 10 % SDS-PAGE reducing gel. Following electrotransfer, the proteins were stained with Ponceau Red. The immunoreactive 14 kDa and non-immunoreactive but colored ~ 4.5 kDa **[D]** polypeptides were excised and submitted to N-terminal sequencing (X represents an undefined residue). **[E]** Mass spectrometric analysis by MALDI-TOF spectrometry of fraction IV. The C-terminal residues sites believed to corresponding to the three ~14 kDa polypeptides are underlined, whereas the expected (potential) cleavage sites are indicated by dashed arrows.

Fig 22 shows the processing of proSKI-1 autocatalytic prosegement candidate sequences by purified, shed SKI-1. The proposed prosegment C-terminal mutant 17 aa peptide IV **[A]** and 15 aa peptide IX **[B]** were digested for 18 h with metal chelation chromatography-purified BTMD-SKI-1. The cleavage products were separated by RP-HPLC using a 5 µm analytical Ultrasphere C18 column (Beckman) as described under "Experimental Procedures". The peptides contained in all but two peaks were identified

by mass spectrometry. The unidentified peaks are attributable to contaminating activities seen in WT/empty vector controls.

Fig. 23 shows the processing of proBDNF and SREBP-2 peptides by BTMD-SKI-1. The 14 aa peptide I **[A]** and 27 aa peptide II **[B]** were digested with BTMD-SKI-1 for 150 and 60 min, respectively. The cleavage products were separated by RP-HPLC using a 5 µm analytical Ultrasphere C18 column (Beckman) as described under "Experimental Procedures". The peptides contained in the major peaks were identified by mass spectrometry and amino acid analysis (*not shown*).

Fig. 24 shows the pH and Ca^{2+} activation profile of BTMD-SKI-1. BTMD-SKI-1 from VV-infected BSC40 cells was assayed as described under "Experimental Procedures" using a binary buffer system consisting of MES and HEPES, along with peptides I or II for the pH profile **[A]**, and peptide II for the Ca^{2+} profile **[B]**. The results represent the average ± SD (indicated as error bars) of three separate determinations.

Fig. 25 is a X-ray film autoradiography showing in situ hybridization pattern for SKI-1 mRNA (A) and APP mRNA (B) at the anatomical plane in sagital section from a 4-day mouse. Note similarity of distribution of SKI-1 and APP. A significant concentration of both SKI-1 and APP mRNA is revealed in the brain (Br), apinal cord (SpC), dorsal root ganglia (DRG), kidney (Ki), skin (Sk) submaxillary gland (SM) and bone tissue (B).

Fig. 26 shows the comparative distribution of SKI-1 and APP in different regions of lacrimal gland of adult male mouse shown by immunocytochemistry. Peripherally located lobes display immunoreaction for both SKI-1 (A) and APP (B) in acinar cells. In the centrally located lobes the immunoreaction for SKI-1 (C) and APP (D) is confined to single cells distributed through the acini (medium arrows) and to intralobular ducts (long arrows).

Fig. 27 illustrates the inhibition of proNGF processing. Rat Schwann cells were infected with either VV:POMC (antigen control), or co-infected with VV:NGF and either VV:POMC (control), VV:PDX, VV:ppFurin or VV:ppPC7. The cells were then pulse-labeled with [^{35}S]Met for 4h and the media immunoprecipitated with an NGF antiserum. The migration positions of the 35 kDa proNGF and the 13.5 kDa NGF are shown.

Fig. 28 illustrates the inhibition of proBDNF processing by furin. Western blot analysis of non-transfected (NT) COS-1 or cells transfected with pcDNA3 recombinants of proBDNF as control (BDNF) or together with recombinants expressing sense (S) or antisense (AS) ppPC7 or ppFurin. The secreted products resolved by SDS-PAGE were analyzed with a BDNF-specific antiserum [Santa Cruz].

Fig. 29 shows the biosynthetic analysis of the fate of the prosegment of SKI-1.
(A) Zymogen processing of [^{3}H] Leu SKI-1 in LoVo cells. LoVo cells overexpressing vaccinia virus full length SKI-1 were pulse-labeled for 15 min with [^{3}H] Leu and then chased for 2h (P15C2h). Cell lysates were immunoprecipitated with antibody to the prosegment, resolved by SDS-PAGE on a 14% gel and the dried gel autoradiographed. The migration positions of the major 26, 24, 14, 10 and 8 kDa prosegments are emphasized.
(B) Zymogen processing of [^{3}H] Leu SKI-1 in BSC40 cells. BSC40 cells overexpressing vaccinia virus SKI-1 prosegment were pulse-labeled for 30 min with [^{3}H] Leu and then chased for 2h (P30C2h). Cell lysates were immunoprecipitated with antibody to the prosegment, resolved by SDS-PAGE on a 14% gel and the dried gel autoradiographed. The migration positions of the 24 and 14 kDa prosegments are emphasized.
Fig. 30 shows the inhibition of hα_4 processing in stable transfectants of Jurkat T cells expressing the mPC5 prodomain mutated at Arg^{84} to Ala. The cell surface proteins of 25 X 10^6 cells were biotinylated and immunoprecipitated with monoclonal hα_4 antibody (HP 2/1). Following SDS gel electrophoresis under reducing conditions and blotting to nitrocellulose the 80 kDa cleavage product was revealed by the chemiluminescence detection of anti-biotin streptavidin horse radish peroxidase.

EXAMPLE 1:

MATERIALS AND METHODS

Polymerase Chain Reaction and Sequencing. Most reverse transcriptase polymerase chain reactions (RT-PCR) were performed using a Titan One Tube RT-PCR system (Boehringer Mannheim) on 1 µg of total RNA isolated from either a human neuronal cell line (IMR-32), mouse corticotrophic cells (AtT20), or rat adrenal glands using a TRIzol reagent kit (Life Technologies). The active site degenerate primers were: His (*sense*) 5'-GGICA(C,T)GGIACI(C,T)(A,T)(C,T)(G,T)(T,G)IGCIGG-3' and Ser (*antisense*) 5'-CCIG(C,T)IACI(T,A)(G,C)IGGI(G,C)(T,A)IGCIACI(G,C)(A,T)GTICC-3' based on the sequences GHGT(H,F)(V,C)AG and GTS(V,M)A(T,S)P(H,V)V(A,T)G, respectively. The amplified 525 bp products were sequenced on an ALF DNA sequencer (Pharmacia). To obtain the full length of rat and mouse SKI-1, we used PCR primers based on the human (12) and mouse sequences, in addition to 5' (13) and 3' (14) RACE amplifications. To avoid errors, at least three clones of the amplified

cDNAs were fully sequenced. The GenBank accession numbers of the 3788 bp mouse mSKI-1 cDNA and 3895 bp rat rSKI-1 are AF094820 and AF094821, respectively.

Transfection and Metabolic Labeling. Human SKI-1 (nt 1-4338) (12) in Bluescript (a generous gift from Dr. N. Nomura, Kazusa DNA Research Institute, Chiba, Japan; gene name KIAA0091, accession No. D42053) was digested with SacII (nt 122-4338) and inserted into the vector PMJ602. The construct was digested with 5' KpnI/3' NheI, cloned into the KpnI/XbaI sites of pcDNA3 (Invitrogen), and the cDNA transfected into HK293 cells with a DOSPER liposomal transfection reagent (Boehringer Mannheim). A number of stable transfectants resistant to G418 and positive on western blots using a SKI-1 antiserum (*see below*) were isolated, and one of them (clone 9), was further investigated. Cells were pulsed for 4h with [^{35}S]Met and the media and cell lysates immunoprecipitated with SKI-1 antisera directed against either amino acids (aa) 634-651, or aa 217-233, or a pro-SKI-1 antiserum directed against the pro-segment comprising aa 18-188 (Fig. 1). Immune complexes were resolved by SDS-PAGE on a 6% polyacrylamide/Tricine gel (15).

Northern Blots, *in situ* Hybridizations and Immunocytochemistry. Northern blot analyses (16) were done on total RNA from adult male rat tissues using either a TRIzol reagent kit (Life Technologies) or a Quick Prep RNA-kit (Pharmacia)and on polyA+ RNA of (male + female) rat adult tissues (Bio/Can Scientific). The blots were hybridized overnight at 68°C in the presence of [^{32}P]UTP SKI-1 cRNA probes, consisting of the antisense of nucleotides 655-1249 of rat SKI-1 (accession No. AF094821). For ISH, the same rat sense and antisense cRNA probes were doubly labeled with uridine and cytosine 5'-{λ-[^{35}S]thio}triphosphate (16). The distribution of SKI-1 mRNA in different tissues of adult and newborn rat (P1) after emulsion autoradiography was investigated. Relative densities of specific SKI-1 mRNA labeling per cell in selected organs have been measured upon counting of silver grains produced by antisense SKI-1 riboprobes and subtraction of non-specific background produced with sense SKI-1 riboprobes. Countings were made under 1000-fold microscopical magnification in the similar regions of adjacent sections stained with hematoxylin and eosin. Results are the mean (S.E.D. of 10 - 16 readings / cell type. Newborn rats were frozen at - 35°C in isopentane and then cut into 14-µm sagital cryostat sections (1, 16). After hybridization, all tissue slides were exposed for 4 or 30 days to X-Ray film or emulsion autoradiography, respectively. For immunofluorescence staining we used a rabbit anti-SKI-1 antiserum at a 1:100 dilution and rhodamine-

labeled goat anti-rabbit IgGs diluted 1:20 (16). Red SKI-1 immunostaining was compared with green staining patterns of both fluorescein-labeled concavalin A (ConA; Molecular Probes, OR), an ER marker, or fluorescein-conjugated wheat germ agglutinin (WGA; Molecular Probes, OR), a Golgi marker (17).

***Ex vivo* and *in vitro* proBDNF Processing.** A vaccinia virus recombinant of human SKI-1 (vv:SKI-1) was isolated as previously described for human proBDNF (vv:BDNF) (15). The vaccinia virus recombinants of the serpins α1-antitrypsin Pittsburgh (α1-PIT; vv:PIT) and α1-antitrypsin Portland (α1-PDX; vv:PDX) (18) were generous gifts from Dr. G. Thomas (Vollum Institute, Portland, OR). For analysis of the cleavage specificity of hSKI-1, 4 x10^6 COS-7 cells were co-infected with 1 pfu/cell of vv:BDNF and either the wild type virus (vv:WT) alone at 2 pfu/cell or with 1 pfu/cell of each virus in the combinations: [vv:SKI-1+vv:WT], [vv:SKI-1+vv:PIT] and [vv:SKI-1+vv:PDX]. At 10h post infection, cells were pulse labeled for 4h with 0.2 mCi [^{35}S]Cys-Met (Dupont). Media and cell extracts were immunoprecipitated with a BDNF antiserum (19; kindly provided by Amgen) at a concentration of 0.5 µg/ml. The precipitates were resolved on polyacrylamide gradient gels (13-22%) and the autoradiograms obtained as described (15). Microsequencing analysis was performed on the [^{35}S]Met-labeled 32 kDa proBDNF and [^{3}H]Leu-labeled 28 kDa BDNF, as described (20). For *in vitro* analysis, the 32 kDa proBDNF obtained from the media of LoVo cells infected with vv:BDNF was incubated overnight with the shed form of SKI-1 obtained from rat Schwann cells (16) co-infected with vv:SKI-1 and vv:PDX, either at different pHs or at pH 6.0 in the presence of selected inhibitors: pepstatin (1 µM), antipain (50 µM), cystatin (5 µM), E64 (5 µM), soya bean trypsin inhibitor (SBTI, 5 µM), 0.5 M phenylmethylsulfonyl fluoride (PMSF) + 50 µM para-aminophenylmethylsulfonyl fluoride (pAPMSF), o-phenanthroline (5 mM) and EDTA (10 mM). The products were resolved by SDS-PAGE on a 15% polyacrylamide gel, transferred to a PVDF membrane and then probed with a BDNF antiserum (Santa Cruz) at a dilution of 1:1000.

RESULTS

Protein Sequence Analysis of SKI-1. We first aligned the protein sequences within the catalytic domain of PC7 (21), yeast subtilases and bacterial subtilisins, together with that of a novel subtilisin-like enzyme from *Plasmodium falciparum* (J-C. Barale *et al., submitted*). This led to the following choice of conserved amino acids around the active sites His and Ser: GHGT(H/F)(V/C)AG and GTS(M/V)A(T/S)P(H/V)V(A/T)G, respectively. Thus, using degenerate oligonucleotides coding for the sense His and antisense Ser consensus sequences we initiated a series of RT-PCR reactions on total RNA (*see Materials and Methods*) and isolated a 525 bp cDNA fragment from the human neuronal cell line IMR-32. This sequence was found to be 100% identical to that reported for a human cDNA called KIAA0091 (Accession No. D42053) obtained from a myeloid KG-1 cell line (12) and 88 % identical to that of a 324 bp EST sequence (Accession No. H31838) from rat PC12 cells. We next completed the rat and mouse cDNA sequences following RT-PCR amplifications of total RNA isolated from rat adrenal glands and PC12 cells, and from mouse AtT20 cells. Starting from the equivalent rat and mouse 525 bp fragments, the complete sequences were determined using a series of RT-PCR reactions with human-based oligonucleotides in addition to 5' (13) and 3' (14) RACE protocols. As shown in Fig. 1, alignment of the protein sequence deduced from the cDNAs of rat, mouse and human SKI-1 revealed a high degree of conservation. Rat and mouse SKI-1 share 98% sequence identity and a 96% identity to human SKI-1. Interestingly, within the catalytic domain (Asp^{218} to Ser^{414}) the sequence similarity between the three species is 100%. Analysis of the predicted amino acid sequence suggests a 17 aa signal peptide, followed by a putative pro-segment beginning at Lys^{18} and extending for some 160-180 amino acids. The proposed catalytic domain encompasses the typical active sites Asp^{218}, His^{249} and Ser^{414} and the oxyanion hole Asn^{338}. This domain is followed by an extended C-terminal sequence characterized by the presence of a conserved growth factor / cytokine receptor family motif **C**^{849}**L**DDSHRQK**DC**F**W**^{861}. This sequence is then followed by a potential 24 aa hydrophobic transmembrane segment and a less conserved 31 aa cytosolic tail that remarkably consists of 35% basic residues. Some of the clones isolated from rat adrenal glands suggested the existence of alternatively spliced rSKI-1 mRNAs in which the segments coding for aa 430-483 or 858-901 are absent. Finally, the phylogenetic tree derived from the alignment of the catalytic domain of SKI-1 with subtilases (22) suggests that it is an ancestral protein that is

closer to plant and bacterial subtilases than to either yeast or mammalian homologues (*not shown*).

Tissue Distribution of SKI-1 mRNA. Northern blot analyses of SKI-1 mRNA in adult male rat tissues reveal that rSKI-1 mRNA is widely expressed and is particularly rich in anterior pituitary, thyroid and adrenal glands (Figs. 2A and 8). A Northern blot of polyA+ RNA obtained from mixed adult male and female rat tissues also showed a wide distribution and a particular enrichment in liver (Fig. 2B). Similarly, analysis of 24 different cell lines (23) revealed a ubiquitous expression of SKI-1 mRNA (*not shown*).

In situ hybridization data obtained in a day 2 postnatal rat also provided evidence of a widespread, if not ubiquitous distribution of rSKI-1 mRNA. Figure 3 shows at the anatomical level the presence of SKI-1 mRNA in developing skin (see also Figure 9), striated muscles, cardiac muscles, bones and teeth as well as brain and many internal organs. Strong hybridization signals were detectable in the retina, cerebellum, pituitary, submaxillary, thyroid and adrenal glands, molars, thymus, kidney and intestine. Evidence for the cellular expression of rSKI-1 mRNA was obtained from analysis of the relative labeling densities per cell in selected tissues, based on a semiquantitative analysis of emulsion autoradiographies (*not shown*). In the central nervous system (CNS) rSKI-1 mRNA labeling was mostly confined to neurons, whereas ependymal cells, supportive glial cells, such as presumed astrocytes, oligodendrocytes, and microglia, exhibited 5-30 fold less labeling/cell (see Table 1 and Figure 10). In addition, within the peripheral nervous system (PNS) trigeminal ganglia reveal a 5-10 fold greater expression in neurons as compared to presumptive Schwann cells (Figures 11 and 12 and Table 1). Labeling was observed in most of the glandular cells in the anterior and intermediate lobes of the pituitary as well as in the pituicytes of the pars nervosa. A semiquantitative comparison in the adult and newborn rat pituitary gland, submaxillary gland, thymus and kidney demonstrated an overall 2-fold decreased labeling of rSKI-1 mRNA with age (*not shown*).

Biosynthesis of hSKI-1. To define the molecular forms of human SKI-1 and their biosynthesis, we generated both a vaccinia virus recombinant (vv:SKI-1) and a stable transfectant in HK293 cells. Three antisera were produced against aa 18-188 (prosegment), 217-233 and 634-651 of SKI-1. Expression of vv:SKI-1 in 4 different cell lines revealed that the enzyme is synthesized as a 148 kDa proSKI-1a zymogen which is processed into 120, 106 and 98 kDa proteins. In this system, both the 148 and 120

kDa forms are recognized by the Pro-domain antiserum, whereas all 4 forms react with the other two antisera. Processing of the 148 kDa proSKI-1a into the 120 and 106 kDa forms occurs in the ER based on the presence of these proteins in cells pre-incubated with the fungal metabolite brefeldin A (see 24 for refs., *not shown*). The same SKI-1-related forms are also observed in stably transfected HK293 cells following a 4h pulse labeling with [^{35}S]Met (Fig.4). The results reveal the intracellular formation of a secretable 98 kDa form (SKI-1s) recognized by both of the SKI antisera but not by the Pro antiserum. These data demonstrate that the 148 kDa proSKI-1a is N-terminally cleaved into an intermediate 120 kDa form containing part of the prosegment (proSKI-1b) which is then further excised to form a non secretable 106 kDa SKI-1. This suggests that two cleavages occur within the prosegment prior to the formation of the presumably membrane-bound 106 kDa form which is later shed into the medium as a 98 kDa soluble SKI-1s.

Intracellular localization of SKI-1. Double staining immunofluorescence was used to compare the intracellular localization of the stably transfected human SKI-1 in HK293 cells and that of either the ER or Golgi markers ConA and WGA (17), respectively. The data show that SKI-1 exhibits: **(i)** peripheral nuclear staining, colocalizing with ConA fluorescence, presumably corresponding to the ER (*not shown*); **(ii)** paranuclear staining colocalizing with WGA fluorescence, suggesting the presence of SKI-1 in the Golgi (Fig. 5A,B) and **(iii)** punctate staining observed in the cytoplasm and within extensions of a few cells (Fig. 5A). Some, but not all of the punctate immunostaining matched that observed with WGA. This suggests that SKI-1 localizes in the Golgi but may sort to other organelles, including lysosomal and/or endosomal compartments. Since in HK293 cells we observed scant immunoreaction to either cathepsin B or cathepsin D (*not shown*), we could not directly assess the presence of SKI-1 within lysosomes. An indication of lysosomal/endosomal localization was provided by the analysis of SKI-1 immunofluorescence within cells pre-incubated for 4h with 10 mM leucine-methyl ester (LME), a specific lysosomal/endosomal protease inhibitor (25). The results showed a net increase in the proportion of cells exhibiting punctate staining (Fig. 5C) as compared to control cells. Thus, SKI-1 immunoreactivity is enhanced upon LME inhibition of lysosomal/endosomal hydrolases.

Enzymatic Activity and Cleavage Specificity of SKI-1. To prove that SKI-1 is a proteolytic enzyme we examined its ability to cleave five different potential precursor substrates. Our choice was based on the tissue expression pattern of SKI-1 (Figs. 2, 3), which led us to select pro-opiomelanocortin (pituitary), pro-atrial natriuretic factor (heart), HIV gp160 (T-lymphocytes) and based on its neuronal expression, pro-nerve growth factor and pro-brain-derived neurotrophic factor (proBDNF). Cellular co-expression of vv:SKI-1 with the vaccinia virus recombinants of each of the above precursors revealed that only proBDNF could be cleaved intracellularly by SKI-1. Thus, upon expression of vv:BDNF alone in COS-7 cells we observed a partial processing of proBDNF (32 kDa) into the known major 14 kDa BDNF product (15), and the minor production of a previously observed (16; Mowla, S.J. *et al., submitted*) but still undefined 28 kDa product (Fig. 6A). Upon co-expression of proBDNF and SKI-1, a net increase in the level of the secreted 28 kDa BDNF is evident, without significant alteration in the amount of 14 kDa BDNF (Fig. 6A). To examine whether the 28 kDa product results from cleavage at a basic residue or at an alternative site, we first co-expressed proBDNF, SKI-1 and either α1-PIT or α1-PDX which are inhibitors of thrombin and PC cleavages, respectively (18, 26). The results show that different from α1-PIT, the serpin α1-PDX selectively blocks the production of the 14 kDa BDNF and that neither α1-PIT nor α1-PDX affect the level of the 28 kDa product. This demonstrates that α1-PDX effectively inhibits the endogenous furin-like enzyme(s) responsible for the production of the 14 kDa BDNF (15), but does not inhibit the ability of SKI-1 to generate the 28 kDa product. Thus, it is likely that the generation of the 28 kDa BDNF takes place via an alternate cleavage. Incubation of the cells with the Ca^{2+} ionophore A23187 abolished the production of both the 14 and 28 kDa products (*not shown*), supporting the notion that similar to the PCs (1-3, 24), SKI-1 is a Ca^{2+}-dependent enzyme.

In Fig. 6B, we present the N-terminal microsequence analysis of [^{35}S]Met-labeled 32 kDa proBDNF and [^{3}H]Leu-labeled 28 kDa BDNF. The sequence of the 32 kDa form revealed the presence of an [^{35}S]Met at position 3 (Fig. 6B), which is in agreement with the proposed sequence of human proBDNF (27) resulting from the removal of an 18 aa signal peptide cleaved at GCMLA^{18}↓APMK site. The N-terminal sequence of the 28 kDa product revealed a [^{3}H]Leu at positions 2, 13 and 14 (Fig. 6B). This result demonstrates the 28 kDa BDNF is generated by a unique cleavage at Thr^{57} in the sequence: RGLT^{57}↓SLADTFEHVIEELL (27).

To prove that SKI-1 is directly responsible for the production of the 28 kDa BDNF at the novel Thr-directed cleavage, we performed *in vitro* studies. Thus, proBDNF was incubated at various pHs with concentrated media of vv:SKI-1-infected Schwann cells. A similar preparation obtained from wild type vaccinia virus-infected cells served as control. The data show that SKI-1 exhibits a wide pH dependence profile revealing activity at both acidic and neutral pHs between pH 5.5 up to 7.3 (Fig. 7A) but also at pH 4.5 and 8 (*not shown*). Analysis of the inhibitory profile of this reaction revealed that metal chelators such as EDTA and o-phenanthroline, or a mixture of the serine proteinase inhibitors PMSF + pAPMSF effectively inhibit the processing of proBDNF by SKI-1. The inhibition by EDTA is expected since like all PCs, SKI-1 is a Ca^{2+}-dependent enzyme. The unexpected inhibition by 5 mM o-phenanthroline may be due to excess reagent since at 1 mM only 25% inhibition is observed (*not shown*). All other class-specific proteinase inhibitors (aspartyl-, cysteinyl-, and serine proteases- of the trypsin-type) proved to be inactive.

Table 1

Tissue	Adult Silver grains/Cell ± SED	Newborn (PI) Silver Grains/Cell ±SED
C.N.S.		
Cerebal Cortex		
Neurons, large	19.7 ± 5.8	ND*
Neurons, medium & small	5.7 ± 2.3	
Astrocytes, presumptive	0.6 ± 0.5	
Hippocampus		ND
Neurons, pyramidal	15.3± 3.9	
Neurons, granules	23.7 ± 5.3	
Corpus callosum		ND
Oligodendrocytes, presumpt.	0.6 ± 0.6	
Spinal cord		ND
Motorneurons	27.8 ± 7.1	
Circumventricular organs		ND
Plexus choroideux	9.6 ± 1.9	
Ependyma (III ventr.)	2.9 ± 0.8	

P.N.S.		ND
Trigeminal ganglion		
Neurons, large	14.6 ± 4	
Satellite cells	3.8 ± 22	
Schwann cells, presumpt.	1.3 ± 1.9	
Pituitary gland		
Anterior lobe cells	4.9 ± 3.6	9.3 ± 2.1
Intermediate lobe cells	4.1 ± 0.9	7.2 ± 1.4
Posterior lobe pituicytes	3.6 ± 3.9	6.7 ± 4.2
Thymus		
Cortical lymphocytes	4.1 ± 0.7	7.1 ± 1.0
Medullary reticular cells	2.7 ± 1.0	4.4 ± 0.9
Adipocytes	0.3 ± 0.6	ND
Fibroblats	0.2 ± 0.1	ND
Submaxillary gland		
Epithelial cells	2.1 ± 1.0	3.9 ± 1.7
Acinar cells	2.4 ± 1.2	4.5 ± 1.7
Kidney		
Glomerular cells	2.8 ± 0.9	4.2 ± 0.9
Convoluted tubules	4.1 ± 2.7	9.8 ± 1.4

*ND = not determined

DISCUSSION

This work provides the first evidence for the existence of a mammalian secretory Ca^{2+}-dependent serine proteinase of the subtilisin-kexin type that selectively cleaves at non-basic residues. Thus, SKI-1 processes the 32 kDa human proBDNF at an KAGSRGLT↓SL sequence generating a 28 kDa form, which may have its own biological activity (Mowla, S.J. *et al., submitted*). Such a cleavage site is close to the consensus site deduced from a large body of work done with the PCs, whereby an **(R/K)**-$(X)_n$-**R** ↓ X-**(L/I/V)**, [where n=0, 2, 4 or 6] motif is favored by most PCs (1-3, 28). Note that in the SKI-1 site, P1 Arg is replaced by Thr and an aliphatic Leu is present

at P2', an amino acid also favored by PCs (1-3, 28). Several proteins are known to be cleaved following Thr. These include human anti-angiogenic platelet factor 4 (6; QCLCVKTT↓SQ) and angiostatin (7; KGPWCFTT↓DP), the neuroendocrine α-endorphin (4; KSQTPLVT↓LF), the ADAM-10 metalloprotease (8; LLRKKRTT↓SA), as well as the amyloidogenic peptide Aβ43 (10; VGGVVIAT↓VI).

Interestingly, comparison of the phylogenetically highly conserved sequence of proBDNF revealed an insertion of hydroxylated amino acids (Thr and Ser) just after the identified SKI-1 cleavage site of human proBDNF. Thus, in rat and mouse proBDNF, two threonines are inserted (RGLTTT—SL) and in porcine proBDNF five serines added (RGLTSSSSS—SL) (27). These observations raised a number of questions: (i) do these insertions affect the kinetics of proBDNF cleavage by SKI-1? (ii) does SKI-1 recognize both single and pairs of Thr and Ser and combinations thereof? (iii) is the presence of a basic residue at P4, P6 or P8 important for cleavage? and (iv) similar to enzymes cleaving at basic residues (29), does the possible phosphorylation at specific Thr or Ser residues affect substrate cleavability by SKI-1? Answers to these questions are provided hereinbelow.

Biosynthetic analysis of the zymogen processing of proSKI-1 demonstrated a two-step ER-associated removal of the pro-segment (Fig. 4). Furthermore, analysis of the [$^{35}SO_4$]-labeled SKI-1 demonstrated only the presence of sulfated 106 and 98 kDa forms but not that of either the 148 or 120 kDa forms recognized by the Pro-segment antiserum (*not shown*). Since sulfation occurs in the *trans* Golgi network, this confirms that the removal of the pro-segment occurs in the ER. Like furin and PC5-B (1-3, 24) the membrane bound 106 kDa SKI-1 is transformed into a soluble 98 kDa form that is released into the medium by an as yet unknown mechanism. The secreted 98 kDa SKI-1s is enzymatically active since it processes proBDNF *in vitro* (Fig. 7). Numerous attempts to sequence the SDS-PAGE purified [^{3}H]Leu and Val-labeled 148 kDa and 98 kDa forms, resulted in ambiguous results, suggesting that SKI-1 is refractory to N-terminal Edman degradation. Presently, we cannot define the two zymogen cleavage sites leading to the sequential formation of the 120 kDa proSKI-1b and 106 kDa SKI-1 deduced by pulse (Fig. 4) and pulse-chase studies (*not shown*). Examination of the pro-segment sequence (Fig. 1), the species-specific proBDNF motif potentially recognized by SKI-1 (*see above*), and the alignment of SKI-1 with other subtilases (22), suggests two possible conserved sites: RNNPSS^{95}↓DYPS and RHSS^{182}↓RRLL.

Both sites predict a cleavage after pairs of Ser with either a P6 or a P4 Arg, respectively.

Phylogenetic structural analysis of the predicted amino acid sequence of SKI-1 reveals that this serine proteinase is closer to plant and bacterial subtilases than it is to yeast and mammalian PCs. The 100% conservation of the catalytic domain sequence, although striking and suggestive of an important function, is not far from the 98% similarity between human and rat PC7 (3, 21). The sequence C-terminal to the catalytic domain of SKI-1 is very different from that of any of the known PCs. In fact, although PCs have a typical P-domain critical for the folding of these enzymes (for reviews see 1-3), we did not find the hallmark sequences (3, 30) of the P-domain within the SKI-1 structure. Instead different from the PCs, we find a conserved growth factor/cytokine receptor motif of which functional importance will need to be addressed, especially since this motif is partly missing in alternatively spliced forms (Fig. 1). Finally, the highly basic nature of the cytosolic tail of SKI-1 (Fig. 1) may be critical for its probable cellular localization within endosomal/lysosomal compartments (Fig. 5), similar to the importance of basic residues for the accumulation of the α-amidation enzyme PAM in endosomal compartments (Milgram, S.L., *personal communication*).

The wide tissue distribution of SKI-1 mRNA transcripts suggests that this enzyme processes numerous precursors in various tissues. Furthermore, the observed developmental down-regulation of the level of its transcripts also suggests a functional importance during embryonic development. The fact that SKI-1 can cleave C-terminal to Thr and possibly Ser residues suggests that, like the combination of PCs and carboxypeptidases E and D (31), a specific carboxypeptidase may also be required to trim out the newly exposed C-terminal hydroxylated residues. Such a hypothesis may find credence in a report suggesting that the amyloidogenic Aβ43 (ending at Thr) may be transformed *in vitro* into Aβ42 and Aβ40 by a brain-specific carboxypeptidase(s) (32).

A recent report demonstrated the existence of a soluble subtilisin-like enzyme exhibiting a 29% sequence identity to SKI-1 in *Plasmodium falciparum* merozoites (PfSUB-1). This enzyme localizes to granular-like compartments and presumably cleaves at a Leu↓Asn bond (33). In that context, SKI-1 may represent the first member of an as yet undiscovered mammalian family of proteinases implicated in the limited proteolysis of proproteins at sites other than basic amino acids that may differ by their intracellular localization and cleavage specificity.

EXAMPLE 2

Genetic and biochemical evidence indicates that SKI-1/S1p is the protease that cleaves sterol-regulatory element-binding proteins (SREBPs) which functions to control lipid biosynthesis and uptake in animal cells { Sakai, J. et al. (1998) Molecular Cell 2, 505-514; Cheng, D. et al. (1999) J. Biol. Chem. 274, 22805-22812; Touré, A. et al. (1999) In: Peptides for the Now Millennium: Proceedings of the 16th American Peptide symposium }. SKI-1 and SREBPs play critical roles in the feedback pathways by which cholesterol suppresses transcription of genes encoding HMG CoA reductase and other enzymes of cholesterol biosynthesis as well as the low density lipoprotein (LDL) receptor. A SKI-1 inhibitor would be of use under clinical conditions in which there is not sufficient down regulation of SREBP dependent transcription by sterols. For example, in the Nieman-Pick group of diseases a high sphingomylin content of cells leads to an increase in proteolysis of SREBP-2 and a subsequent increase in cholesterol biosynthesis { Scheek, S. et al. (1997) Proc. Natl. Acad. Sci. USA 94, 11179-11183; Spence, M.W., and Callahan, J.W. (1989) Spingomyelin-cholesterol lipidoses: The Nieman-Pick Group of Diseases. *In The Metabolic Basis of Inherited Disease* (Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., editors), McGraw-Hill Publ. Co., 6th edition, chapter 66, 1655-1675; Svirirodov, D. (1999) Histology & Histopathology 14 (1): 305-319 }. Perhaps of greater significance, nuclear SREBP-1c protein levels were significantly elevated in mouse models for non-insulin dependent diabetes, *ob/ob* and aP2 SREBP-1c mice, which was associated with elevated mRNA levels for known SREBP target genes involved in the biosynthesis of fatty acids (Shimomura, I. *et al.* J. Biol. Chem.1999; 274:30028-30032).

In addition, the inhibition of the SREBP- dependent transcription of farnesyl diphosphate synthase, like HMG-CoA reductase and farnesyl-protein transferase inhibitors, by inhibition of farnesyl pyrophosphate biosynthesis could potentially be useful to treat a number of diseases such as Ras-dependant cancers and restenosis (Reference - United States Patent 5,925,651). With regard to a potential treatment for restenosis, HMG-CA reductase inhibitors decrease smooth muscle (SMC) cell migration and proliferation, and induce SMC apoptosis { Bellosta, S. et al. (1998) Atherosclerosis 137, S101-S109; Guijarro, C. et al. (1998) Circulation Research 83, 490-500 }.

As mentioned above, inhibition of PC activity seems to offer new therapeutical targets. Unfortunately, previous attempts using inhibitory peptides have failed either

due to cytotoxicity of used agents or poor targeting [17;18]. We have focused on the inhibitory properties of PC prosegments in order to find a safe and effective way for enzyme silencing.

To study the effect of the SKI-1 prosegment (ProSki-1) on the SREBP processing and mediated transcriptional activity we isolated a cDNA fragment covering the 188 amino acids that make up the signal peptide and the prosegment of SKI-1 including the predicted cleavage site RRLL[176]. This autocatalytic cleavage site was confirmed by mass spectral analysis and amino acid sequencing by other investigators [19]. We isolated stable cell lines overexpressing SREBP-1 (neo resistance) and ProSki-1 plasmid (zeo resistance). A background SREBP-1 overexpression was used in order to improve detection of nuclear NH_2-terminal segment of SREBP in immunoblot experiments.

The effect of ProSki-1 on target gene mRNA: mRNA expression in HK293 cells was studied by Northern blotting as described in the methods section. In wild type (wt), vector only, and SREBP overexpressor cells in presence of lipids the mRNA levels were low for all studied genes: LDL-receptor, HMG-CoA reductase, farnesyl diphosphate (FDP) (Fig. 13), and fatty acid synthase (FAS) (Fig. 14). However, when these cells were treated with media containing no cholesterol a clear increase was observed in mRNA expression for all these genes, as demonstrated in earlier studies. Interestingly, corresponding mRNA levels were greatly reduced in both conditions in cells overexpressing ProSKI-1 and SREBP-1 suggesting that SREBP mediated transcription can be blocked efficiently by the prodomain mediated inhibition of the SKI-1 protease (Figs. 13 and 14). The effect was observed in early passages of previously frozen cell lines. However, when the same clones were kept in culture for future passages, in contrast to earlier findings the target gene mRNA levels were now normal or even higher than in control cells. (Figure 15). This finding suggests that cells can adapt to new conditions and maintain their lipid homeostasis even without SREBP mediated regulation and synthesis. This finding was supported in another experiment with several cell lines overexpressing SREBP-1 or SREBP-1 and ProSki-1 (Fig. 16). While HMG CoA reductase and farnesyl diphosphatase varied markedly between different cell lines containing only SREBP-1 (Fig. 16, lanes 1-5), mRNA levels measured from cells overexpressing ProSki-1 and SREBP-1 (Fig. 16, lanes cl4, cl6, and cl9) showed no variation and were higher than in SREBP-1 cells.

The ff ct of ProSki-1 on nuclear SREBPs: Western blot experiments were performed to illustrate the effect of ProSKI-1 on SREBP-1 processing in these cells. After staining with an antibody against the NH_2-terminal end of SREBP-1 a band around 60 kDa appeared on blots of nuclear extracts (Fig. 17), as demonstrated earlier by other investigators [2,3]. As expected, only a weak signal was detected in presence of sterols. In absence of sterols a significant increase was observed, especially in SREBP-1 cells. Only minute amounts of nuclear SREBPs were detected when ProSKi-1 was present suggesting that sterol mediated proteolysis of SREBPs is efficiently blocked in these cells in presence of ProSki-1 (Figure 17 shows the data from clones 6 {lane 5} and 9 {lane 6}).

The inhibitory effect of ProSKI-1 was further demonstrated by studying the processing of cytoplasmic full length SREBP-1 (proSREBP-1) (Fig. 18). The processing of proSREBP-1 by SKI-1 / S1P into intermediate (intSREBP-1) forms shown previously by other investigators[19], was clearly demonstrated in clones overexpressing SREBP-1. Significantly, in cell lines overexpressing SREBP-1 together with the inhibitory prodomain of SKI-1 (pSKI + SRE) accumulation of the proSREBP-1 was observed and formation of the intermediate form(s) of SREBP-1 was abolished. These results, along with the observed reduction in nuclear SREBP (Fig. 17), indicate that ProSKI-1 efficiently inhibits SKI-1 protease activity and blocks SREBP processing in mammalian cells. In addition, the specificity of ProSKI-1 inhibition was studied by using a substrate not processed by SKI-1 (neurotrophin-3; NT-3). Both the level and furin-derived processing of NT-3 were unaffected by the presence of ProSKI-1 (*not shown*). These results suggest that ProSKI-1 is SREBP- and pro-BDNF- specific and that it does not affect other secretory proteins which are not substrates for SKI-1.

In these experiments a pro-domain was successfully used for the first time as a subtilase inhibitor *in vivo*. ProSki-1 seems to be a promising therapeutical tool for SREBP-mediated pathologies, which may or may not be directly related to cholesterol or fatty acid homeostasis. For instance SREBP-dependent isoprenoids, such as farnesol and geranylgeraniol, have been shown to associate e.g. with endothelial nitric oxide synthetase (eNOS) [20-23], vascular smooth muscle proliferation and migration as well as ras-protein mediated cell proliferation [24-28]. Furthermore, links to PPAR-γ mediated signaling system including adipocyte differentiation and insulin resistance have already been reported [29-33]. This novel prosegment approach to inhibit enzyme activity will certainly also inspire other investigators in different fields, since it may be

possible to specifically inhibit other enzymes with this prosegment technology leading to new treatments for a variety of diseases. On the other hand, these results provide new data supporting the existence of an SREBP-independent, but lipid dependent (Fig. 3) control of the lipid homeostasis in human cells, although the alternative sensor of lipids under these conditions is currently unknown.

Materials and Methods

Materials:

Cell Culture: HK293 cells were maintained as monolayers in Dulbecco's modified Eagle's medium containing 100 units / ml penicillin and 100 µg / ml streptomycin sulfate (medium A) supplemented with 10 % fetal calf serum. 24 hours before RNA and protein extractions medium A was supplemented with 5 % lipoprotein deficient serum, 50 µM mevalonate (Sigma), 50 µM compactin (Sigma) and with no sterols or 1 µg/ml of 25-hydroxy-cholesterol and 10 µg/ml of cholesterol. 4 hours before protein extraction 25 µg/ml N-acetyl-leucinyl-leucinyl norleucinal was added. Total RNA was isolated with Trizol (Gibco BRL) reagent according to the instructions of the manufacturer. In order to extract proteins cells were washed and collected in PBS with protease inhibitors (). After addition of buffer A (Triton x 100 1 %,50 mM tris maleate, 2 mM $CaCl_2$, inhibitor coctail (), and ALLN) cells were mixed with pipette and allowed to swell on ice for 20 minutes Then the solution was centrifuged for 5 minutes at 15 000 rpm and supernatants representing membrane proteins were collected and stored until analyzed at -70 °C. Remaining pellets were resuspended in Buffer B (20 mM Tris pH 7.9, 400 mM NaCl, 1mM EDTA, 1mM EGTA, and protease inhibitors). Samples were shaken at 4 C for 1 hour and centrifuged and the supernatant was frozen in aliquots at -70 °C.

Plasmid constructions: SKI-1 prosegment containing aa 1-188 was isolated by PCR using following oligonucleotides: [5' GGA TCC GAA GAA ACA TCT GGG CGA CAGA 3'] and [5' CTC GAG GGC TCT CAG CCG TGT GCT 3'] and cloned into PCR 2.1 TA cloning vector for sequencing. After that it was subcloned into the $pcDNA_{3zeocin}$ vector (Invitrogen) (BamHI / HindIII sites) for transfections.

SREBP-1 in bluescript IISK (ATCC 79810) subcloned into SalI / BamHI sites of the $pcDNA_{3geneticin}$.

Transf ctions: HK293 cells were plated at a density of $5x10^5$ / 60 mm dish in medium A with 10 % fetal calf serum and were cultured until they were 40-60 % confluent. The cells were then transfected with 10 µg plasmid DNA ($pcDNA_{3neo}$, $pcDNA_{3neo}$-SREBP-1,

$pcDNA_{3neo}$-SREBP-1 and pcDNA $_{3zeo}$ProSKI-1) using Lipofectin reagent (Life Technologies, city, state) according to manufactures instructions. On day two medium containing appropriate selection agents (800 µg/ml Geneticin for $pcDNA_{3neo}$, x00 µg/ml Zeocin for $pcDNA_{3zeo}$) were added. The medium was changed every two days until defined colonies were evident. Colonies were isolated and formed stable cell lines were analyzed by immunoblotting with ProSKI-1 and SREBP-1 antibodies.

Northern blotting: 20 µg of total RNA was electroforetically separated in an 1.0 % agarose gel, and transferred to Hybond N^+ filters (Amersham, city, state) by capillary blotting. After transfer filters were crosslinked by UV irradiation in a Stratalinker (Stratagene). Filters were prehydridized at 42 °C for 1 hour and hybridized with random labeled ^{32}P cDNA probes for 16-20 hours. Ultrahyb buffer (Ambion) was used. After hybridization filters were washed and exposed to film for indicated time and bands were quantified by densitometry. Following primer pairs were used to clone cDNA probes: HMG CoA reductase [5' GAG GAA GAG ACA GGG ATA AAC 3'] [5' GGG ATA TGC TTA GCA TTG AC 3'], farnesyl diphosphate [5' AGC CCT ATT ACC TGA ACC TG 3'], [5' GAA TCT GAA AGA ACT CCC CC 3'], Fatty acid synthase [5' TTC CGA GAT TCC ATC CTA CG 3'], [5' TGC AGC TCA GCA GGT CTA TG 3'], Acetyl CoA carboxylase [5' TCT CCT CCA ACC TCA ACC AC 3'], [5' CCA GCC TGT CAT CCT CAA TAT C 3'], SREBP-1 [5' GGA GCC ATG GAT TGC ACT TTC 3'], [5' AGG AGC TCA ATG TGG CAG GA 3'], LDL-receptor [5' 3'], [5' 3']. Amplification products were cloned into pGEM (Promega) and sequenced. 18S cDNA was purchased from Ambion.

Immunoblot analysis: 50 µg of nuclear extract and membrane fractions were separated in an SDS-PAGE gel. After electrophoresis proteins were transferred to a nitrocellulose membrane. Membranes were stained with appropriate primary SREBP-1 (Santa Cruz), ProSki-1 and secondary antibodies. After washing chemiluminescent substrate (Santa Cruz) was added, and membranes were exposed to x-ray film for 1-30 min. Gels were calibrated with prestained molecular weight markers (New England Biolabs).

EXAMPLE 3

The soluble SKI-1 isoform, collected from cell media, was used to study the *in vitro* cleavage properties of this enzyme on a number of synthetic substrates. In addition, we present data on the *in vitro* inhibitory character of three prosegment

constructs of SKI-1, which we obtained as bacterial recombinant proteins. Moreover, we examined the processing of hSKI-1 in LoVo cells infected with a VV recombinant as well as in a stable transfectant of HK293 cells (10).

EXPERIMENTAL PROCEDURES

Vaccinia Virus Recombinant of BTMD-SKI-1 - The preparation of a soluble form of hSKI-1 involved the initial amplification by polymerase chain reaction (PCR) of a 1250 base pair (bp) product encompassing nucleotides (nts) 491-1740 of the hSKI-1 cDNA (12), which includes the initiator methionine. The sense (s) and antisense (as) oligonucleotides were 5' GTGACCATG-AAGCTTGTCAACATCTGG 3' and 5' ACACTGGTCCCTGAGAGGGCCCGGCA 3', respectively. This completely sequenced fragment, which had been inserted into the PCR2.1 TA cloning vector (Invitrogen), was first digested with NotI and AccI. It was then ligated with the similarly digested full-length hSKI-1 cDNA 3.5 kb product, resulting in a product called 5' hSKI-1-FL. In order to obtain a soluble form of hSKI-1 with a hexa-His sequence just before the stop codon, PCR amplification was carried out using the sense and antisense oligonucleotides: 5' ATTGACCTGGACAAGGTGGTG 3' and 5' G G A T C C T C T A G A T C A G T G G T G G T G G T G G - TGGTGGTGCTCCTGGTTGTAGCGGCCAGG 3'. This resulted in a 165 bp fragment encoding the C-terminal sequence PGRYNQE^{997}-(H_6)* (10). Following digestion with 5' EcoNI and 3' XbaI, the product was ligated to the aforementioned and similarly digested 5' hSKI-1-FL. This cDNA, coding for BTMD-SKI-1 ending with a hexa-His sequence, was then transferred to the BamH1/XbaI site of the (VV) transfer vector PMJ601. A recombinant was then isolated as previously reported (13). The VV recombinant of full-length hSKI-1 has been described (10).

Biosynthetic Analyses - Seventeen hours following infection with 2 pfu each of VV:SKI-1 and VV:BTMD-SKI-1 recombinants, human LoVo cells (3 x 10^6) were radiolabeled with 500 µCi of [^{3}H]Leu for 2h or pulsed for 15 min followed by a chase of 2h, in the presence or absence of 5 µg/ml of the fungal metabolite brefeldin A (BFA) as described (10,14). Media and cell lysates were immunoprecipitated with SKI-1 antiserum directed against either aa 634-651, or the prosegment comprising aa 18-188 (10). Immune complexes were resolved by SDS-PAGE on an 8% or 14% polyacrylamide/Tricine gel (10) and the dried gels autoradiographed (10,14). All biosynthesis experiments were p rformed at least twice.

Isolation and Purification of Recombinant hSKI-1 Prosegments - Three N-terminal fragments of hSKI-1 were isolated by PCR using a common (s) oligonucleotide [5' GGATCCGAAGAAACATCTGGGCGACAGA 3'] and one of three (as) oligonucleotides [5' CTCGAGGGAGAGGCTGGCTCTTCG 3'], [5' CTCGAGGGCTCTCAGCCGTGTGCT 3'] or [5' CTCGAGTGTCTGGGCAACCTGGCGCGGG 3']. These prosegment fragments, ending at aa 169, 188, and 196 (10), were cloned in the PCR 2.1 TA cloning vector for sequencing. Then they were transferred into the BamHI / XhoI sites of the bacterial expression vector pET 24b (Novagen). These recombinants were transformed into the *E. Coli* strain BL21. Protein expression was induced with 1mM isopropyl β-D-thiogalactoside and the cultures were grown for 3h at 37°C. The cell pellets were sonicated on ice in a binding buffer containing 6M guanidine-HCl (Novagen) until a clear solution was obtained. The clarified and filtered solution was then applied to a nickel affinity column (Novagen) and eluted with 500 mM imidazole. The eluates were dialyzed overnight at 4°C against 50 mM sodium acetate (pH 7). The protein precipitate was solubilized with glacial acetic acid, filtered through a 0.45 µm disk and further purified on a 5 µm C4 column (0.94 x 25 cm; Chromatographic Sciences Company Inc; CSC) by reverse-phase high performance liquid chromatography (RP-HPLC). The purity was assessed by Coomassie staining and the identity of the products verified by mass spectrometry on a Matrix Assisted Laser Desorption Time of Flight (MALDI-TOF) Voyageur DE-Pro instrument (PE PerSeptive Biosystems). The amounts of prosegments were determined by quantitative amino acid analysis (13).

Expression and Purification of Recombinant BTMD-SKI-1 - Following infection of BSC40 cells (75 x 10^6 cells) with 2 pfu/cell of recombinant VV:BTMD-SKI-1, the cells were washed and incubated at 37 °C for 18h in a serum-free minimal essential medium (MEM; Life Technologies). Media (45 ml) were then dialyzed, concentrated 20-fold to 2.2 ml on Centriprep-30's (Amicon) and stored at –20 °C in 40 % glycerol. For purification[2], the concentrated media were applied to a Ni^{2+} affinity resin (Novagen) or a Co^{2+} affinity resin (Clontech Laboratories) as described by the manufacturer. After two washes with 5 mM imidazole, the protein was eluted with 200 mM imidazole and tested for enzymatic activity and immunoreactivity by Western blot (*see below*).

Western Blot Analyses - Aliquots of partially purified BTMD-SKI-1 were separated by 8 or 12 % SDS-PAGE followed by electro-transfer of the proteins onto polyvinylidene fluoride (PVDF) membranes (Schleicher and Schuell). These

membranes were probed with an antiserum directed against either SKI-1 [aa 217-233 (Ab:*N*) or aa 634-651 (Ab:*S*)] or pro-SKI-1 [(aa 18-188 (Ab:*P*)]. Protein bands were visualized by enhanced chemiluminescence (ECL) (Boehringer Mannheim).

Purification, N-terminal Sequencing and Mass Spectrometric Analysis of the Secreted Recombinant Prosegment(s) of hSKI-1 - Concentrated media obtained from either VV:BTMD-SKI-1 infected BSC40 cells or from a stable transfectant of full-length hSKI-1 in HK293 cells (10) were loaded onto an RP-HPLC 5 µm C4 column (0.94 x 25 cm) (Vydac). Proteins were eluted at 2 ml/min using a 1 %/min linear gradient (15-70 %) of 0.1 % aqueous trifluoroacetic acid (TFA)/CH_3CN with monitoring at 210 nm. The products were analyzed by Western blotting, after which the immunoreactive fractions were further purified on a CSC 5 µm C4 column (0.2 x 25 cm). Mass values were obtained by MALDI-TOF spectrometry using the [1]matrix 3,5 dimethoxy-4-hydroxycinnamic acid (Aldrich Chemical Co). For N-terminal sequencing, fraction IV proteins (Fig. 21A) were separated by SDS-PAGE, transferred to Immobilon-P membranes, and stained with Ponceau Red. The 14 and 5 kDa bands were excised and sequenced using an Applied Biosystems Model 477 sequenator operating in the gas-phase mode (15).

Synthesis of Peptide Substrates - All Fmoc amino acid derivatives (L-form), the coupling reagents, and the solvents for peptide synthesis were purchased from PE Biosystems Inc. (Framingham, Mass, USA), Calbiochem (San Diego, Ca, USA), or Richelieu Biotechnologies (Montréal, QC, Canada). The various linear synthetic peptides and internally quenched fluorogenic (Q-) substrates reported in this article are: (I) **hproBDNF(50-63)**: KAGSRGLTSLADTF, (II) **hSREBP-2(504-530)**: GGAHDSDQHPHSGSGRSVLSFESGSGG, (III) **hSKI-1(174-191)**: WHATGRHSSRRLLRAIPR, (IV) **hSKI-1(174-188+LE)**: WHATGRHSSRRLLRALE, (V) **hSKI-1(182-188+LE)**: SRRLLRALE, (VI) **hSKI-1(156-172)**:WQSSRPLRRASLSLGSG, (VII) **hSKI-1(187-201)**: RAIPRQVAQTLQADV; (VIII) **hSKI-1(128-136)**: PQRKVFRSL; (IX) **hSKI-1(128-142)**: PQRKVFRSLKYAESD; (X) **Q-hSKI-1(132-142)**: Abz-

[1] Although we managed to produce limited quantities of partially purified SKI-1 using metal chelating resins, there was insufficient enzyme to carry out full kinetic analyses. However, since the medium of WT virus-(or control vector)-expressing cells produced no significant peptide hydrolysis (with the exception of peptides VIII and IX), we mainly used the concentrated media of BSC40 cells infected with VV:BTMD-SKI-1. Thus, the metal chelation-purified enzyme served mainly to verify that the enzyme from concentrated media behaved similarly to this form. We therefore confirmed all of the peptide cleavage sites, the SREBP-2 pH optimum, and the Ca^{2+} requirement presented below.

VFRSLKYAESD-Y(NO_2)-A; (XI) **Q-hSKI-1(134-142)**: Abz-RSLKYAESD-Y(NO)-A. Except for the first two peptides, which were purchased from the Sheldon Biotechnology Institute (McGill University, QC, Canada), all other peptides were synthesized with the carboxy-terminus in the amide form. Peptides III-XI were prepared on a solid phase peptide synthesizer (Pioneer model, PE Biosystems) using either 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluophosphate (HBTU) / N-hydroxybenzotriazole (HOBT) or HATU (O-[7-azabenzotriazol-1-yl]-N,N,N',N'-tetramethyluronium hexafluorophosphate) / diisopropyl ethyl amine (DIEA)-mediated Fmoc chemistry with PAL-PEG unloaded resin and the standard side chain protecting groups (16). For the incorporation of the two unnatural amino acids [Abz and Y(NO_2)], an extended coupling cycle was used instead of either the standard or fast cycles.

Purification, Analysis, and Digestion of Peptide Substrates - The crude peptides were purified by RP-HPLC using a semi-preparative CSC-Exsil C18 column (2.5 x 25 cm). Monitoring at 210 nm, the peptides were eluted with a 1 %/min linear gradient (5 % to 60 %) of aqueous 0.1 % TFA/CH_3CN at 2 ml/min and. The peptide purity and concentration were determined by quantitative amino acid analysis (16). The identity of each purified peptide was confirmed by MALDI-TOF spectrometry using the matrix α-cyano 4-hydroxycinnamic acid (Aldrich Chemical Co).

For digestions, each peptide was typically reacted at 37 °C with 10 µl of the concentrated enzyme preparation in a buffer consisting of 50 mM HEPES (N-2-Hydroxyethyl Piperazine-N'-2 EthaneSulfonic acid) (ICN Biomedicals Inc), 50 mM MES (2-[N-Morpholino] EthaneSulfonic acid) (Sigma Chem Co.), and 3 mM Ca^{2+}-acetate (pH 6.5). The digestion products were separated by RP-HPLC on a Beckman 5 µm Ultrasphere C18 column (0.2 x 25 cm) and eluted with a 1 %/min linear gradient of aqueous 0.1 %TFA/CH_3CN (5-45 %) at a flow rate of 1 ml/min. The collected peptides were characterized by mass spectrometry and amino acid composition, which was also used to quantitate the amount of various substrates and products. The digestions of the quenched fluorogenic peptides were analyzed by RP-HPLC using a dual UV (210 nm) and fluorescence (excitation and emission wavelengths of 320 and 420 nm, respectively) detector (Rainin).

pH Optimum, Calcium-Dependence and Inhibitor Profile - The protocols used were essentially the same as reported previously (13). Stocks of the buffer described above were adjusted to pH 5.0-8.5 at 0.5 unit increments by addition of either acetic acid or sodium hydroxide. In order to investigate the calcium requirement of SKI-1,

increasing concentrations of Ca^{2+}-acetate were used ranging from 0 to 10 mM. For inhibition studies, the enzyme in the reaction buffer was preincubated with the desired agents for 30 min prior to addition of peptide II.

$K_{m(app)}$, $V_{max(app)}$ and $K_{i(app)}$ determinations - Following digestion reactions with increasing substrate concentrations, the products were separated by RP-HPLC. The rate of substrate hydrolysis was obtained from the integrated peak areas of the chromatograms. $K_{m(app)}$ and $V_{max(app)}$ values were estimated using nonlinear regression analysis (Enzfitter software; Elsevier Biosoft, Cambridge, UK) of plots of the hydrolysis rate *vs* the substrate concentration. For apparent inhibitor constant [$K_{i(app)}$] determinations, variable inhibitor concentrations within the range of 15-70 % inhibition were used at three concentrations of peptide IV ranging from 0.6 to 3.5 times the $K_{m(app)}$ value. The $K_{i(app)}$ values were estimated from Dixon plots as described (16). For the two quenched peptides, kinetic parameters were determined as described (17).

RESULTS

SKI-1 Overexpression, Purification, Biosynthesis, and Prosegment Processing

We have previously shown that overexpression of full-length SKI-1 (FL-SKI-1) in HK293 cells results in shedding of a 98 kDa form (sSKI-1) of this enzyme into the medium (10). Based on this finding, we engineered a soluble form of SKI-1 (BTMD-SKI-1), ending at residue 997, to which we added a hexa-His sequence at the C-terminus (Fig. 19A). In a comparative biosynthetic analysis, shown in Fig. 19B, LoVo cells were infected with the SKI-1 virus constructs VV:FL-SKI-1, VV:BTMD-SKI-1, and wild type virus (VV:WT). After labeling the cells for 3h with [^{35}S]Cys, proteins in the media were immunoprecipitated with an antiserum directed against either the prosegment of SKI-1 (Ab:*P*) or an internal SKI-1 sequence (Ab:*S*). In both cases, a protein of ~14 kDa co-immunoprecipitated with the 98 kDa sSKI-1 or the 100 kDa BTMD-SKI-1 (bSKI-1, Fig. 19B) that was not seen with VV:WT infections. Since Ab:*P* was raised against a recombinant SKI-1 prosegment peptide and has been shown previously to detect the SKI-1 zymogen (10), we concluded that the ~14 kDa peptide is most likely derived from the cleaved prosegment (the full-length prosegment is ~24 kDa – *see below*). The fact that it co-immunoprecipitated with the enzyme under denaturing conditions suggests a strong interaction between SKI-1 and this region of its prosegment. The actual stoichiometry of enzyme-to-prosegment is not clear from this experiment, since it was carried out using two different antisera and denaturing conditions. We also observed that some of the 100 kDa BTMD-SKI-1 is cleaved into

a 98 kDa form similar to that found with FL-SKI-1 (Fig. 19B). This conversion is presumably carried out by endogenous "shedding enzymes" (10,18) that can act on both forms of SKI-1, although C-terminal sequencing would be needed to confirm this hypothesis.

Western blot analyses of media now obtained from BSC40 cells infected with VV:BTMD-SKI-1 also revealed a secreted ~100 kDa immunoreactive band (Fig. 19C). The same band was detected using either an antiserum against the N-terminal region of the SKI-1 catalytic domain (Ab:*N*) or one against a more C-terminal region (Ab:*S*). When Ab:*P* was mixed together with Ab:*S* and used to probe the metal affinity column-purified SKI-1 preparation (indicated by the * in Fig. 19C), we were able to again detect the ~14 kDa prosegment fragment, further supporting our hypothesis that it forms a strong association with the enzyme. (It should be noted that although a mixture of Ab:*S* and Ab:*P* was used in order to detect both proSKI-1 and BTMD-SKI-1 simultaneously, when either Ab:*N* or Ab:*S* were used alone, only the 100 kDa or 14 kDa species were observed, respectively (*not shown*)).

In order to evaluate the rate of zymogen processing and the fate of the prosegment, LoVo cells overexpressing VV:FL-SKI-1 were pulse-labeled with [^{3}H]Leu for 15 min and then chased for 2h. Figure 20 shows an SDS-PAGE analysis of the cell lysates immunoprecipitated with Ab:*P* (left panel). At least five immunoreactive polypeptides (molecular masses of ~26, 24, 14, 10 and 8 kDa) which were not present in controls infected with VV:WT, were detected. In order to further define in which organelle(s) this processing occured, LoVo cells infected with VV:FL-SKI-1- were pulse-labeled with [^{3}H]Leu for 2h in the presence or absence of BFA (Fig. 20, right panel). In both cases, the same five major, intracellular, immunoreactive prosegment forms could still be detected. Since the fungal metabolite BFA is known to disassemble the Golgi complex and cause the ER to fuse with the *cis, medial* and *trans* Golgi (but not the *trans* Golgi network, TGN) (19), this result strongly implies that the initial zymogen processing of proSKI-1 occurs early along the secretory pathway. Possible locations include the ER or *cis* Golgi, as was previously reported (10). Moreover, further processing of the prosegment into yet smaller fragments also occurs in these organelles.

To further characterize the prosegment of SKI-1, we took advantage of a stable transfectant of FL-SKI-1 in human HK293 cells that we had made previously (10). This system has the added advantage that the possibility of VV overexpression artifacts

influencing the processing of the prosegment is eliminated. Concentrated culture medium from these cells (serum-free) was purified via RP-HPLC using first a semi-preparative C4 column (*not shown*) followed by an analytical C4 column (Fig. 21A). The eluted fractions were analyzed by Western blot using Ab:*P* (Fig. 21B). Immunoreactive peptides ranging from ~4.5-24 kDa were apparent. N-terminal sequencing of the very abundant ~14 kDa protein in fraction IV (Fig. 21C) revealed a major sequence starting at Gly^{18} of pre-proSKI-1 (10,12). This clearly defines the signal peptidase cleavage site as LVVLLC^{17}↓GKKHLG, which is one aa before that predicted by signal peptidase cleavage site algorithms (10,11). The N-terminal sequence of the ~4.5 kDa polypeptide (Fig. 21D) revealed that it starts at Pro^{143}, indicating a cleavage at the sequence KYAESD^{142}↓PTVPCNETRWSQK. This fragment is most likely the product of cleavage between Asp and Pro that may be caused by the acidic conditions encountered in either RP-HPLC, Edman sequencing (20), or sample preparation for SDS-PAGE analysis (21). An unexpected benefit of this cleavage was our finding that phenylthiohydantoin (PTH)-Asn^{148}, which occurs in the putative N-glycosylation site AsnGluThr was readily detected in this sequence. Thus, the predicted N-glycosylation site Asn^{148} within the prosegment of SKI-1 is not employed, at least in this expression system. This conclusion was also supported by the prosegment's resistance to endo H and endo F digestion (*not shown*). Of the two eukaryotic subtilases known to contain a potential N-glycosylation AsnGluThr site, *i.e.* kexin (22) and SKI-1 (10), it appears that at least the latter's prosegment is not N- glycosylated. Finally, the separation of the above prosegment fragments from mature SKI-1 using RP-HPLC (Fig. 21A,B) and non-reducing SDS-PAGE (*not shown*), suggests that none of the Cys residues in the prosegment (10) are linked by disulfide bridges to the rest of the enzyme.

As a preliminary means of characterizing the SKI-1 prosegment fragments, MALDI-TOF analysis (Fig. 21E) of fraction IV from Fig. 21B was carried out. Three major molecular ions of masses 13,351, 13,518, and 13,685 Da were detected, with an expected error of ± 25 Da for this mass range. Combined with the previous N-terminal sequencing results of the ~14 kDa peptide (Fig. 21C), these mass values indicate that this peptide has heterogeneous C-termini that are derived from cleavages near the sequence **RK**VFRSL**K**^{137}, as indicated in Fig. 21E. In fact this region contains three potential SKI-1 cleavage sites (8) with an R or K at the P4 position and either an F, R or K at the P1 position. Although the calculated molecular masses of 13,339, 13,496 and 13,696 for the polypeptides G^{17}KK---**RK**VF^{133}, G^{17}KK---**RK**VF**R**^{134} and G^{17}KK---

RKVF**R**SL[136], respectively, match within experimental error (± 22 Da) the observed masses in Fig. 21E, these assignments should only be taken as a first indication (*see below*). Moreover, the predicted G[17]KK---**RK**VF**R**SL[136] fragment does not correspond to the expected SKI-1 cleavage motif of a basic residue at the P4 position. Hence, this secreted peptide could result either from cleavage at G[17]KK---**RK**VF**R**SL[136] or, more likely, at G[17]KK---**RK**VF**R**SL**K**[137] Lys[137] followed by basic carboxypeptidase cleavage of the C-terminal Lys (23). Since we were unable to obtain consistent mass spectra of the ~4.5 kDa polypeptide that was sequenced in Fig. 21D, we could not use this technique to approximate its C-terminus, which presumably corresponds to the C-terminus of the processed SKI-1 pro-segment. We therefore resorted to synthetic peptide cleavage as a tool to accurately define potential prosegment cleavage sites.

Analysis of Synthetic Prosegment-derived Peptide Cleavages - Based on our detection of ~26and 24 kDa SKI-1 prosegment products (Fig. 20), as well as on a mutagenesis study of SREBP-2 cleavage sites (8), we synthesized three SKI-1 prosegment peptides encompassing potential, C-terminal, autocatalytic cleavage sites (10,11). All contain Arg at P4 and either Leu, Lys, Ala or Phe at P1 (peptides III, VI and VII shown in Table II-A). Of these peptides containing only native sequences, the only one with detectable cleavage by SKI-1-containing concentrated medium (from either VV:BTMD-SKI-1-infected BSC40 cells or SKI-1 transfected HK293 cells) was peptide III (WHATGRHSS**R**RL**L**[186]↓RAIPR) (see Table II-A). No cleavages were observed when VV:WT-infected or empty vector-transfected media were used (*not shown*). Metal chelation chromatography-purified enzyme further supported that this cleavage is effected by SKI-1 (Fig. 22A; peptide III), and the products were positively identified via mass spectrometry.

Similarly, based on the mass spectrometry data in Fig. 21E, we synthesized two peptides (VIII and IX) encompassing the putative internal processing site(s) of the SKI-1prosegment. Both were cleaved at multiple locations by SKI-1-containing concentrated medium from HK293 transfectants (*not shown*). Further analysis revealed that one of these cleavages, corresponding to PQ**R**KVF[133]↓**R**SL, was as prevalent in empty vector-transfected HK293 medium as in SKI-1-transfected medium (see. Table III-A, peptide VIII). In contrast, the PQRKVF**R**SL**K**[137]↓ YAESD cleavage was only seen in SKI-1-containing medium. This cleavage was also confirmed using metal chelation chromatography-purified enzyme (Fig. 22B; peptide IX) and mass spectrometry to identify the products. However, also clearly visible are the PQ**R**KVF[133]↓**R**SL**K**YAESD

cleavage products. We acknowledge that there could be residual contaminating proteases in our purified SKI-1 preparations (minor bands were visible on colloidal gold-stained membranes of SKI-1 preparations). Thus, while we are confident that SKI-1 cleaves its prosegment at the C-terminal WHATGRHSS**R**RL**L**^{186}↓RAIPR site and at the internal PQ**R**KVF**R**SL**K**^{137}↓YAESD site, our data do not allow us to rule out SKI-1-mediated cleavage at the PQ**R**KVF^{133}↓**R**SL**K**YAESD site.

Comparing the simple cleavage rates of the SKI-1 prosegment internal and C-terminal sites, we observed that the former was vastly superior to the latter (*not shown*). We also noticed that the peptides best processed by SKI-1 contain an acidic residue at the P3' or P4' substrate site, whereas those that did not appeared to be cleaved poorly or not at all (Table III-A). Moreover, we had previously established that SKI-1 does not cleave the fluorogenic peptides RGLT-MCA, RGLTT-MCA and RSVL-MCA (10), which lack P' residues. Based on these observations, we asked if replacing the Ile and Pro residues at P3' and P4' of the C-terminal prosegment processing site would significantly improve the SKI-1-mediated cleavage of peptide III. Thus, we synthesized two mutants of this peptide (peptides IV and V, the latter truncated by 8 aa at the N-terminus) in which the Ile and Pro residues at P3' and P4' were replaced by Leu and Glu, respectively. As shown in Table II-B, this change significantly improved the processing of these peptides, such that we were able to determine $V_{max(app)}$ / $K_{m(app)}$ values. The approximately two-fold difference in these values for peptides IV and V further suggests that determinants N-terminal to the P4 position may also play a role in substrate specificity. The SKI-1 specificity of these peptide cleavages was also verified using metal chelation chromatography-purified enzyme (when VV:WT-infected or empty vector-transfected media were used, no peptide processing was observed).

In Vitro Kinetic Properties of SKI-1: Comparative Analysis of Synthetic Peptide Cleavages - In a previous report (10), sSKI-1 was shown, to cleave the 32 kDa proBDNF into a 28 kDa form at the **R**GL**T**↓SL sequence *in vitro* with a pH optimum close to neutrality. Similar to PCs (1-3), we suggested that SKI-1 might be a Ca^{2+}-dependent enzyme since the calcium ionophore A23187 inhibited the *ex vivo* cleavage of proBDNF (10). In order obtain kinetic analyses of defined SKI-1 substrates, we examined a 14 aa peptide spanning the hproBDNF processing site (10), K^{50}AGS**R**GL**T**↓SLADTF^{63} (peptide I) and a 27 aa hSREBP-2-related peptide (8), G^{504}GAHDSDQHPHSGSG**R**SV**L**↓SFESGSGG^{530} (peptide II). Concentrated SKI-1-

containing medium (from either VV:BTMD-SKI-1-infected BSC40 cells or SKI-1 transfected HK293 cells) was reacted with these peptides at pH 6.5, followed by MALDI-TOF mass spectrometric analysis of the RP-HPLC-purified products. The expected cleavages were confirmed and did not occur using WT-/empty vector-derived media (Fig. 23). Again, the metal chelation chromatography-purified enzyme generated the same products as the concentrated media (not shown). We then demonstrated that the optimal pH and calcium concentrations for efficient cleavage of the hSREBP-2 peptide (II) are pH 6.5 and 2 mM Ca^{2+}, respectively (Fig. 24). Interestingly, the pH optimum observed with the the proBDNF peptide (I) is sharper than that obtained with peptide II. In the former case, the enzyme still retains about 30% of its activity at pH 5.0 and 55 % of its activity at pH 8.5 (Fig. 24A). Similar results for the pH optimum of peptide II cleavage were obtained with metal chelation-purified BTMD-SKI-1 (*not shown*). In contrast, however, the pH optimum of peptide IX with the purified enzyme was 8.0, with no activity detectable below pH 5.5.

A summary of the kinetic analyses of the synthetic proBDNF (peptide I) and SREBP-2 (peptide II) cleavages by SKI-1 is shown in Table II-B. Both peptides are cleaved at comparable kinetic efficiencies with $V_{max(app)}$ / $K_{m(app)}$ values of 0.002 and 0.004 h^{-1}, respectively. In comparison, the $V_{max(app)}$ / $K_{m(app)}$ value estimated with peptide IV is 5-10-fold higher than those obtained with peptides I and II (Table II-B). The N-terminal truncation of peptide IV from 17 to 9 aa (peptide V, Table II-A) caused a 4-fold reduction in catalytic efficiency (Table II-B).

Table III shows the inhibitor profile of SKI-1, in which it is clear that this enzyme is quite sensitive to metal chelators such as EDTA and to the calcium chelator EGTA. In addition, the transition metals Cu^{2+} and Zn^{2+}, but not Ni^{2+} or Co^{2+}, inhibit the enzyme at mM concentrations. As reported using the 32 kDa proBDNF (10), assays with the synthetic SREBP-2 peptide demonstrated that the metal chelator *o*-phenanthroline becomes inhibitory at concentrations above 1 mM. The other non-chelator inhibitors tested had minimal or no effects on SKI-1 activity.

In order to develop a convenient *in vitro* assay for SKI-1, we designed a number of internally quenched fluorogenic substrates and tested their cleavage efficacy by SKI-1. The two best peptides encompassed the processing site RSLK↓ within the hSKI-1 prosegment (peptides X and XI, Table II-A). Mass spectrometric analysis confirmed that both peptides were cleaved at the RSLK↓ site by shed SKI-1 derived from HK293 cell transfects, but not by medium obtained from HK293 empty vector transfectants.

This processing generated the fluorescent N-terminal peptides Abz-VFRSLK or Abz-RSLK, and a non-fluorescent C-terminal peptide YAESDY(NO_2)-A (*not shown*). Measurements of kinetic parameters demonstrated that peptides X and XI are about 3- and 16-fold better substrates than the C-terminal prosegment peptide IV (Tables I-B and III), suggesting that the shorter peptide XI may be the best SKI-1 substrate tested to date. This cleavage was completely abolished in the presence of 10 mM EDTA, in agreement with the Ca^{2+}-dependence of SKI-1 activity (Fig. 24B).

SKI-1 Inhibition by its Prosegment -_One important question remaining is whether the SKI-1 prosegment functions as an inhibitor of its enzymatic activity, analogous to the prosegments of other subtilases (3). We thus prepared prosegment constructs, designated ending near the proposed C-terminal processing site RRLL^{186} (Fig. 22A): PS1; extending to Leu^{169}; PS2, extending to Ala^{188}; and PS3, extending to Leu^{197} To each C-terminus we coupled a hexa-His tag. These prosegment constructs were expressed in bacteria and purified by Ni^{2+}-chelation chromatography followed by RP-HPLC (*see Experimental Procedures*). The purity of these prosegments was confirmed by SDS-PAGE/Coomassie staining and aa analysis (*not shown*). A summary of the inhibitory potency of each prosegment using peptide IV as a substrate is shown in Table V. Kinetic analysis using Dixon plots (15) indicated a competitive inhibition mechanism (*not shown*). Although PS2 exhibits the best apparent inhibitory constant ($K_{i(app)}$ = 97 nM), PS3 ($K_{i(app)}$ = 127 nM) and PS1($K_{i(app)}$ = 182 nM) are similarly potent SKI-1inhibitors. When PS2 was digested with carboxypeptidase B to eliminate the His-tag, its inhibitory potency was not affected (*not shown*), confirming that this tag is not responsible for the observed inhibition. We also tested the inhibitory activity of the RP-HPLC-fractionated native prosegment (see Fig. 21). Only, the material from fraction IV, which included the full-length ~24 kDa prosegment, was inhibitory, whereas that of the others , including the ~14 kDa peptide alone or in combination with smaller fragments, were not inhibitory (*not shown*).

DISCUSSION

Limited proteolysis of inactive precursor proteins at sites marked by paired or multiple basic residues is a widespread process (1,2). Less common is the recent finding that bioactive peptides or proteins can also be generated by limited proteolysis after either hydrophobic or small residues (3). SKI-1 represents the first mammalian member of subtilisin-like processing enzymes with such substrate specificity (10,11). It is a widely expressed enzyme (10) that may play a crucial role in cholesterol and

fatty acid metabolism (11). Due to its very recent discovery, information regarding its enzymatic properties, substrate specificity, and the function of its proregion have only begun to be addressed.

Many peptidyl hydrolases, including subtilases, possess a prodomain which acts both as an intramolecular chaperone and a highly potent inhibitor of its associated protease (24,25). Activation of the enzyme typically requires release of the prosegment in an organelle-specific manner. For furin (26) the release occurs in the TGN, whereas for PC1 and PC2 (27) it occurs in immature secretory granules. The data presented in this report demonstrate that SKI-1 is unique among the mammalian subtilases, since both the C-terminal and internal cleavages of its prosegment occur in the ER. Hence, this enzyme does not appear to require an acidic environment for activation, assuming, by analogy with other subtilases (3), that prosegment release is the crucial step leading to zymogen activation. We propose the following sequence of events presumably leading to SKI-1 activation: **1)** The signal peptide is removed in the ER by a signal peptidase cleavage at LVVLLC^{17}↓GKKHLG (Fig. 21C). **2)** The prosegment is processed into a non-N-glycosylated polypeptide with an apparent molecular mass of ~24-26 kDa (Fig. 20). **3)** This prosegment is further processed into 14, 10 and 8 kDa intermediates (Fig. 20). While these multiple cleavages may be catalyzed by SKI-1 itself, the participation of other proteases cannot be excluded. The major cleavages leading to the formation of the ~24 and ~14 kDa products occur within 10 min, and the other secondary ones within 30 min (*not shown*). Since treatment of cells with BFA did not significantly alter these processing events, they most likely occur in the ER (Fig. 20). It is possible that the generation of prosegment fragments from the ~24-26 kDa pro-form leads to a loss of inhibition in a fashion similar to that of subtilisin E (24,25). Indeed, our results demonstrate that while the full-length prosegment is inhibitory, its ~14 kDa product is not. Surprisingly, some pro-region-derived polypeptides are found associated with SKI-1 in cell culture media. Thus, in contrast to furin (26), the low pH and high Ca^{2+} concentrations prevailing in the TGN do not lead to propeptide dissociation. High ionic concentrations (up to 1M NaCl) such as those used in immunoprecipitation (Fig. 19B) and metal chelation protein purification (Fig. 19C) also do not disrupt the complex. It is only during RP-HPLC purification (Fig. 21A), in the presence of strong acids and organic solvents, that the prosegment peptides dissociate from SKI-1. These data suggest that hydrophobic interactions may be critical, as is the case for subtilisin (24,25).

To distinguish the SKI-1 prosegment autoprocessing sites (C-terminal and internal) from several closely situated candidate sites, we employed a combination of mass spectrometry and synthetic peptide digestion. In the case of the C-terminal site, only one of three candidate peptides (III) was processed by SKI-1 (Table II-A), indicating that **R**RL**L**^{186}↓RAIP is the most likely autoprocessing site. For the internal site, preliminary mass spectrometric data suggested three distinct cleavages occuring within the sequence PQ**R**KVF**R**SL**K**YAESD^{142} (Fig. 21E). Two of the three possible sites (PQ**R**KVF^{133}↓**R**SL**K**YAESD and PQ**R**K**V**FR^{134}↓SL**K**YAESD) appeared to satisfy the proposed SKI-1 recognition motif requiring a P4 basic residue (8). The third possibility (PQ**R**K**V**F**R**SL^{136}↓**K**YAESD) could be considered by assuming the cleavage actually occurred at PQ**R**KVF**R**SL**K**^{137}↓YAESD, followed by endogenous, basic carboxypeptidase removal of the C-terminal Lys residue (23). Assays carried out *in vitro* with synthetic peptides corresponding to this region of the prosegment (peptides VIII and IX) produced the same cleavage products (*not shown*), but only the PQ**R**KVF**R**SL**K**^{137}↓YAESD cleavage was unique to SKI-1. Thus, we propose that the aforementioned site is the most likely internal autoprocessing site, with the qualification that PQ**R**KVF^{133}↓**R**SL**K**YAESD may occur to a lesser extent (see Results and Fig. 22).

Other information regarding the substrate preferences of SKI-1 was obtained by replacing the P3' and P4' Ile and Pro residues of the C-terminal cleavage site peptide (III) by Leu and Glu (peptides IV and V) to create a very well processed SKI-1 substrate. While it would appear that the presence of an acidic residue at P4' significantly enhances the rate of substrate hydrolysis, it is also possible that the presence of Pro at P4' hinders efficient substrate processing. The presence of similar acidic residues at the P3' or P4' position of the two confirmed substrates of SKI-1 (peptides I and II) as well as in the prosegment internal cleavage site RSLK^{137}↓YA**E**S (Table II-A) lends support to the first argument. In addition to these residues, others also appear to play a role in SKI-1 substrate cleavage catalysis. The peptide pairs IV/V and X/XI both point to influences of positions N-terminal to the P4 residue. Interestingly, the efficiency of the truncated C-terminal peptide V is lower than that of peptide IV, whereas that of the truncated internal (quenched) peptide XI is higher. Taken together, these data indicate the importance of aa at both the P and P' positions in SKI-1-mediated substrate hydrolysis.

The data presented in Fig. 24 indicate that SKI-1 functions most efficiently near neutral pH and at 2-3 mM Ca^{2+}. This is in general agreement with the conditions that

reportedly prevail in the ER (28,29). However, closer examination of the data reveal that the pH optimum of SREBP-2 cleavage (peptide II, Fig. 24A) is actually 6.5, an observation that we confirmed using our purified SKI-1 preparation (*not shown*). This suggests that the processing of SREBP might occur outside of the ER, perhaps in the Golgi where pH values of ~6.5 have recently been reported (30,31). Indeed, there is now cellular evidence suggesting that SREBP cleavage may occur in the Golgi rather than in the ER (32,33). The pH optimum of SKI-1 appears to be dependent on the substrate employed; proBDNF (10) and its related peptide (I), appear to be well cleaved even at pH 5.5, suggesting that it could cleave this (and possibly other substrates) in acidic endosome-like compartments where it was previously localized (10). On the other hand, cleavage of the internal, autocatalytic, prosegment processing site PQ**RK**VF**R**SLK^{137}↓YAESD (Fig. 22B) is optimal at pH 8 (*not shown*), implying that this event, as we concluded from our biosynthesis assays, takes place most effectively in the ER. Overall, the pH and Ca^{2+} profiles of SKI-1 resemble those of the constitutively secreted PCs (1,13). The inhibitor profile of SKI-1 (10, Table III), showing that enzymatic activity is significantly inhibited by EDTA, EGTA and only high concentrations of *o*-phenanthroline, tend to discount the likelihood that SKI-1 is a transition metal-dependent proteinase. In fact, SKI-1 activity is inhibited by low concentrations of certain transition metals, such as Cu^{2+} and Zn^{2+}.

Directed by the observation that peptides containing the primary processing site of the prosegment of PC1 are potent inhibitors of its activity, and that the C-terminal basic residues of furin and PC7 are essential for enzyme inhibition (34,35), we assessed the inhibitory potency of three SKI-1 recombinant propeptides. All of these end at sequences near the RRLL^{186}RA cleavage site. Interestingly, the three prosegments displayed comparable inhibitory potencies (Table V). Compared to proPC1 (34), pro-furin and proPC7 (35), the $K_{i(app)}$ values (Table V) are up to 250 fold higher. This suggests that the prosegment of SKI-1, although potentially inhibitory *in vivo*, may function more as a chaperone, catalyzing the productive folding of SKI-1. Indeed, since SKI-1 may be active in the ER (10,11), whereas the PCs are not (13,26), the lower inhibitory potency of the prosegment of SKI-1 may be adapted to the conditions prevailing in this cellular compartment. In the case of PCs, highly effective inhibition by the prosegment may be needed in order to ensure that these enzymes are activated only when they reach the TGN or secretory granules (1-3). The 14 kDa fragment, which represents the major secreted form of the prosegment, is tightly

associated with SKI-1 (Fig. 19C) yet it is not inhibitory (*not shown*). Accordingly, this segment may serve a chaperonin-like function similar to that reported for the N-terminal 150 aa of 7B2 towards proPC2 (36,37).

Two articles describing the processing, purification and *in vitro* activity of hamster SKI-1/S1P were published (38,39). On most points, our results are in close agreement with those recently published. Thus, these authors characterized the processing of the SKI-1/S1P prosegment, proposing that the ER is the major site of autocatalytic activation of SKI-1 at the same cleavage sites as we present here. They also went on to purify a soluble form of the enzyme, showing that it correctly processes SREBP-2 derived peptides as well as a 16 residue peptide spanning the internal prosegment cleavage site. In addition, they find that cleavage of fluorogenic RSLK-MCA peptide derived from the same sequence is optimal at ~3 mM Ca^{2+} at slightly alkaline pH. Discrepancies such as the lack of detectable shed SKI-1/S1P, multiple secreted prosegment forms, and a different signal peptidase site can most likely be attributed to the different cell types and species employed in the two studies.

In conclusion, the present work firmly establishes that SKI-1 is a Ca^{2+}-dependent subtilase with a reasonably neutral pH optimum, depending on the substrate employed.We also demonstrate that SKI-1 can cleave substrates C-terminal to Thr, Leu and Lys residues, thus providing direct, *in vitro* evidence that it is a candidate converting enzyme responsible for the generation of 28 kDa proBDNF (10) and SREBP-2 processing at site 1 (11). For efficient cleavage, it appears that substrates should contain a basic residue at P4 and an aliphatic one at P2 (Table II-A). Furthermore, aa at the P3' and P4' positions seem to exert an important discriminatory effect. The best substrate tested so far is the quenched flurorogenic substrate Abz-RSLK _ YAESDY(NO_2), thereby providing a convenient and sensitive assay for SKI-1 activity. The present data demonstrate that only the full length SKI-1 prosegment is inhibitory. Thus, overexpression of this prosegment in cell lines may provide a novel method for inhibiting the cellular activity of this enzyme in a fashion similar to the that of over-expressed profurin and proPC7 (35). Finally, it is anticipated that precursor substrates other than the sterol regulating SREBPs (8) and the neurotrophin proBDNF (10) will be identified, thereby extending the spectrum of activity of this unique and versatile enzyme.

Table II-A

Synthetic peptide substrates

Peptides were first reacted with approximately equal quantities of BTMD-SKI-1 medium for 2-18 h as described in "Experimental Procedures". When cleavage was not detected, a 10-fold concentrated enzyme preparation was tested. Arrow thickness is a qualitative estimate of the cleavage efficacy.

Peptide	P16	P12	P8	P4	P1	P4'	P8'
I			**K** A G S **R** G L **T** _ S L A **D** T F				
II	G G A H D S D Q H P H S G S G **R** S V **L** _ S F **E** S G S G G						
III		W H A T G **R** H S S **R** R L **L** ↓ R A I P R					
IV		W H A T G **R** H S S **R** R L **L** _ R A L **E**					
V				S **R** R L **L** _ R A L **E**			
VI [1]		W Q S S **R** P L R **R** A S **L** - S L G S G					
VII [1]			**R** A I P **R** Q V **A** - Q T L Q A **D** V				
VIII [2]			P Q **R** K V **F** - R S L				
IX [2,3]		P Q **R** K V F **R** S L **K** _ Y A **E** S **D**					
X			Abz-V F **R** S L **K** _ Y A **E** S **D** Y(NO_2)-A				
XI			Abz-**R** S L **K** _ Y A **E** S **D** Y(NO_2)-A				

[1] No cleavage detected even with a 10-fold excess of enzyme.

[2] Cleavage detected but not attributable to SKI-1.

[3] Kinetic determinations of this peptide were not attempted due to the presence of multiple cleavages.

Table II-B

Kinetic constants for the hydrolysis of peptide substrates by BTMD-hSKI-1

Increasing concentrations of peptides were reacted with identical quantities of BTMD-SKI-1 medium for times chosen to produce 5-30 % substrate hydrolysis. Data analysis was carried out as described in "Experimental Procedures". The values are averages of duplicate assays.

Peptide	$K_{m(app)}$ (nM*1000)	$V_{max(app)}$ (nmol/h)	$V_{max(app)} / K_{m(app)}$ ($h^{-1} L^{-1}$)
I	169	0.4	0.002
II	124	0.5	0.004
IV	17	0.4	0.023
V	109	1.1	0.010

Table III

Effect of selected protease inhibitors on BTMD-hSKI-1 activity

Digestion reactions using BTMD-SKI-1 medium plus peptide II were carried out as described in "Experimental Procedures". The agents were preincubated with the enzyme for 30 min.

Inhibitor	Concentration (mM)	Hydrolysis of SREBP-2 peptide (% of control) [1]
Control	-	100
APMSF	1.0	95
PMSF	1.0	85
TPCK	1.0	71
TLCK	1.0	100
SBTI	0.5 [2]	100
Cystatin	0.01	100
Antipain	1.0	100
Chymostatin	1.0	100
Leupeptin	1.0	100
Pepstatin	0.1	97
E-64	0.01	100
O-Phenanthroline	0.05	135
	1.0	90
	5.0	0
EDTA	10.0	0
EGTA	10.0	15
Dithiothreitol	10.0	92
$CuSO_4$	1.0	0
$ZnSO_4$	1.0	0
$NiSO_4$	1.0	93
$MgCl_2$	1.0	100
$CoCl_2$	1.0	100

[1] Values represent averages of duplicate assays (variation is $\pm$ 5 %).

[2] Concentration in mg/ml.

Table IV

Kinetic constants for the hydrolysis of quenched fluorogenic substrates by shed-hSKI-1

Assays and data analysis were carried out as described in Table II-A. The values are averages of duplicate assays.

Peptide	$K_{m(app)}$ (μM)	$V_{max(app)}$ (μmoles/h)	$V_{max(app)} / K_{m(app)}$ ($h^{-1} L^{-1}$)
X	31.3	34.0	1.1
XI	8.7	56.9	6.5

Table V

Effect of pro-segment peptide constructs on BTMD-hSKI-1 activity

Digestion reactions using BTMD-SKI-1 medium plus peptide IV were carried out as described in "Experimental Procedures". The prosegment peptides were preincubated with the enzyme for 30 min. Values were deduced from the Dixon plots obtained from three separate experiments.

Pro-segment construct	$K_{i(app)}$ (nM)
PS1	182.0 ± 0.5
PS2	97.5 ± 4.5
PS3	127.3 ± 6.2

EXAMPLE 4

SIMILARITY OF ANATOMICAL DISTRIBUTION OF SKI-1 mRNA TO THAT OF APP

β-amyloid precursor protein (β-APP) is a member of a highly conserved gene family, which includes amyloid precursor-like protein-1 and amyloid precursor-like protein-2 { McNamara, M.J. et al. (1998) Brain Research 804, 45-51; Rassoulzadegan, M. et al. (1998) The EMBO Journal 17, 4647-4656 }. Mammalian subtilases, exemplified by SKI-1, may be responsible for limited cleavage at hydrophobic residues present in biologically important precursor proteins such as ß-amyloid precursor protein (ß-APP) (TableVI). SKI-1 has recently been identified as the enzyme which cleaves sterol-regulatory element-binding protein (SREBP) in a fashion analogous to the ß-secretase cleavage of APP { Sakai, J. et al. (1998) Molecular Cell 2, 505-514 }. The cleavage of SREBP by SKI-1 (Site 1 protease) at a position 20 residues to the lumenal side of the first membrane-spanning segment is analogous to the β-secretase cleavage of β-APP at a position 28 amino acids from the membrane { Brown, M.S. and Goldstein, J.L. (1997) Cell 89, 331-340 }.

Similarity of anatomical distribution of SKI-1 mRNA to that of APP suggests a functional link between both proteins.

In situ hybridization performed in 4-day-mouse provides evidence of a similar distribution of mRNA coding for the membrane proteins SKI-1 and APP (Fig. 25). Their spatial distribution was observed to be significantly overlapping within different tissues such as brain and spinal cord, cranial and spinal ganglia, submaxillary gland, thymus, kidney, bones, skin and many other. Their mRNA distribution was partially similar to that of two other proteases, namely the convertase furin and the peptidase neprilysin. A much different distribution was observed with convertases PC1, PC2 and PC5. It is clearly established that an increase in cellular cholesterol levels results in the inhibition of activity of SKI-1 / S1P { reviewed in Edwards, P.A., and Ericsson, J. (1999) Annu. Rev. Biochem. 68, 157-185 }. In a similiar fashion, an increase in dietary cholesterol leds to significant reductions in brain levels of secreted APP derivatives, including sAPPα, sAPPβ, Aβ1-40 and Aβ1-42 { Howland, D.S. et al. (1998) J. Biol. Chem. 273, 16576-16582 }. The nature of the relationships between cholesterol, SKI-1 and APP metabolism are complex.

Cellular association between SKI-1 and APP in lacrimal gland. Potential use of shed SKI-1 in tears as diagnostic tool.

Results of immunocytochemistry performed in mouse lacrimal glands provides evidence for the presence of SKI-1 and APP in the same cells types, including intralobular duct epithelial cells and some acinar cells (Fig. 26). The finding of SKI-1 in the lacrinal gland suggests the possibility of developing a diagnostic assay analyzing tears; perhaps based on two -dimensional polyacrylamide gel electrophoresis for disease diagnosis { Molley, M.P. et al. (1997) Electrophoresis 18, 2811-2815; Glasson, M.J. et al. (1998) Electrophoresis 19, 852-855; Grus, F.H., and Augustin, A.J. (1999) Electrophoresis 20, 875-880; Iskeleli, G. et al. (1999) Electrophoresis 20, 875-880 }.

TABLE VI

PRECURSOR CLASSIFICATION BASED ON HYDROPHOBIC AND/OR SMALL AMINO ACID CLEAVAGE

Precursor protein	Cleavage site sequence P8 P7 P6 P5 P4 P3 P2 P1 P1' P2' P3' P4' P5' P6' P7' P8'
(h) proBDNF	**Lys**-Ala-Gly-Ser-**Arg**-Gly-**Leu**-**Thr**|Ser-Leu-Ala-Asp-Thr-Phe-Glu-His
(r) proBDNF	**Lys**-Ala-Gly-Ser-**Arg**-Gly-**Leu**-**Thr**|Thr-Thr-Ser-Leu-Ala-Asp-Thr-Phe
(h) proSKI-1	**Arg**-His-Ser-Ser-**Arg**-Arg-**Leu**-**Leu**|Arg-Ala-Ile-Pro-Arg-Gln-Val-Ala
	Arg-Lys-Val-Phe-**Arg**-Ser-**Leu**-**Lys**|Tyr-Ala-Glu-Ser-Asp-Pro-Thr-Val
	Thr-Pro-Gln-Arg-**Lys**-Val-**Phe**-**Arg**|Ser-Leu-Lys-Tyr-Ala-Glu-Ser-Asp
	Val-Thr-Pro-Gln-**Arg**-Lys-**Val**-**Phe**|Arg-Ser-Leu-Lys-Lys-Tyr-Ala-Glu
(h) SREBP-2	Ser-Gly-Ser-Gly-**Arg**-Ser-**Val**-**Leu**|Ser-Phe-Glu-Ser-Gly-Ser-Gly-Gly
(h) SREBP-1a	**His**-Ser-Pro-Gly-**Arg**-Asn-**Val**-**Leu**|Gly-Thr-Glu-Ser-Arg-Asp-Gly-Pro
(r) pro-Relaxin (B-chain)	Ala-Ser-Val-Gly-**Arg**-Leu-**Ala**-**Leu**|Ser-Gln-Glu-Glu-Pro-Ala-Pro-Leu
(h) pro-CCK (CCK5)	**Arg**-Ile-Ser-Asp-**Arg**-Asp-**Tyr**-**Met**|Gly-Trp-Met-Asp-Phe-Gly-Arg-Arg
(r) pro-Somatostatin (Antrin)	Asp-Pro-**Arg**-Leu-**Arg**-Gln-**Phe**-**Leu**|Gln-Lys-Ser-Leu-Ala-Ala-Ala-Thr
(b) Chromogranin A (82|83)	Leu-Leu-**Lys**-Glu-Leu-Gln-Asp-**Leu**|Ala-Leu-Gln-Gly-Ala-Lys-Glu-Arg
(b) Chromogranin A (309|310)	Met-Ala-**Arg**-Ala-Pro-Gln-**Val**-**Leu**|Phe-Arg-Gly-Gly-Lys-Ser-Gly-Glu
(b) Chromogranin B (629|630)	Glu-Leu-Glu-Asn-Leu-Ala-Ala-**Met**|Asp-Leu-Glu-Leu-Gln-Lys-Ile-Ala
(b) Chromogranin B (634|635)	Ala-Ala-Met-Asp-Leu-Glu-**Leu**-**Gln**|Lys-Ile-Ala-Glu-Lys-Phe-Ser-Gly
(r) pro-Renin	**Lys**-Ser-Ser-Phe-Thr-Asn-**Val**-**Thr**|Ser-Pro-Val-Val-Leu-Thr-Asn-Tyr
(r) α-Endorphin	**Lys**-Ser-Gln-Thr-Pro-Leu-**Val**-**Thr**|Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys
(r) γ-Endorphin	Ser-Gln-Thr-Pro-Leu-Val-**Thr**-**Leu**|Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn
(r) pro-AVP (CPP)	Gly-Pro-Ala-Arg-**Glu**-Leu-**Leu**-**Leu**|Arg-Leu-Val-Gln-Leu-Ala-Gly-Thr
(h) ADAM-10 (kuzbanian)	Leu-Leu-**Arg**-Lys-**Lys**-Arg-Thr-**Thr**|Ser-Ala-Glu-Lys-Asn-Thr-Cys-Gln
(h) β-APP	
β-Secretase site	Glu-Glu-Ile-Ser-**Glu**-Val-Lys-**Met**|Asp-Ala-Glu-Phe-Arg-His-Asp-Ser
β-Secretase site (*Swedish*)	Glu-Glu-Ile-Ser-**Glu**-Val-**Asn**-**Leu**|Asp-Ala-Glu-Phe-Arg-His-Asp-Ser
$\beta\varepsilon_1$-Secretase site	Ile-Ser-Glu-Val-**Lys**-Met-Asp-**Ala**|Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr
$\beta\varepsilon_2$-Secretase site	Glu-Phe-**Arg**-**His**-**Asp**-**Ser**-**Gly**-**Tyr**|Glu-Val-His-His-Gln-Lys-Leu-Val

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EXAMPLE 5

Prodomains in general (for example furin and PC7 prodomains) function *in trans* when expressed in mammalian cells to inhibit their cognate subtilisn-like convertase

We have recently shown that the prosegment of furin expressed as an independent domain (preprofurin, ppfurin) can specifically inhibit neurotrophin processing. In these assays, successful inhibition requires not only that the prodomain enter the secretory pathway, but that it remain there long enough to interact with the target PC (most likely furin within the TGN). Figures 27 & 28 depict vaccinia virus constructs or transient transfections of prosegments preventing the maturation of the neurotrophins NGF and BDNF in Schwann or COS-1 cells, respectively. The modest inhibition with the prodomain of PC7 (ppPC7) is most likely due to inhibition of furin, since PC7 is a poor effector of proNGF and proBDNF maturation in these cells. The complementary experiment to demonstrate selectivity by the prosegment of PC7 will be carried out once we are able to establish unique *in vivo* PC7 substrates.

Most proteases from the four major classes (thiol, aspartic, serine, and metallo) are synthesized as inactive precursor molecules with N-terminal extensions (prosegments) that play critical roles in folding, stability and regulation of enzymatic activity { Khan, A.R., and James, M.N. (1998) Protein Sci. 7, 815-836 }. The proregions of the PCs have been shown to function as potent inhibitors of their cognate enzymes *in vitro*. We present data for the first time showing that the expression of a prosegment as an independent domain in a cell-based (ex vivo) assay functions as a PC inhibitor (Figs. 27 and 28). In these assays, successful inhibition requires not only that the prodomain enter the secretory pathway, but that it remain there long enough to interact with the target PC (most likely furin within the TGN).

We have shown that expression of full length SKI-1 prosegment (22-24 kDa with sequence ending at the secondary cleavage sequence RHSSRRLL) inhibits SKI-1 activity in stable HK 293 cell lines (Example 2). However, since the prodomain of SKI-1 is processed at an internal primary cleavage site RKVFRSLK to give a 14 kDa N-terminal fragment (Fig. 29A&B) we predict that mutation of this site will generate an even more effective SKI-1 inhibitor. In fact, in the case of the mouse PC5 prodomain we have shown that mutation of the internal prosegment cleavage site does in fact generate a inhibitor of integrin $h\alpha_4$ 150 kDa processing to 80kDa and 70kDa species (Fig. 15).

EXAMPLE 6

SKI-1 Peptide Substrates for fluorescence resonance energy transfer (FRET) – Based Proteolysis Assays

A large number of synthetic peptides based on potential cleavage sites in the hSKI-1 prodomain, proBDNF and the loop region of SREBP-2 were synthesized. These are:

(i) hSKI-1 (156-172)

Trp-Gln-Ser-Ser-Arg-Pro-Leu-Arg-Arg-Ala-Ser-Leu↓Ser-Leu-Gly-Ser-Gly

(ii) hSKI-1 (174-191)

Trp-His-Ala-Thr-Gly-Arg-His-Ser-Ser-Arg-Arg-Leu-Leu↓Arg-Ala-Ile-Pro-Arg

(iii) hSKI-1 (174-188+Leu+Glu)

Trp-His-Ala-Thr-Gly-Arg-His-Ser-Ser-Arg-Arg-Leu-Leu↓Arg-Ala-Leu-Glu

(iv) hSKI-1 (181-188+Glu)

Ser-Ser-Arg-Arg-Leu-Leu↓Arg-Ala-Ile-Glu

(v) hSKI-1 (187-201)

Arg-Ala-Ile-Pro-Arg-Gln-Val-Ala↓Gln-Thr-Leu-Gln-Ala-Asp-Val

(vi) hSKI-1 (128-136)

Pro-Gln-Arg-Lys-Val-Phe-Arg-Ser-Leu

(vii) hSKI-1 (128-142)

Pro-Gln-Arg-Lys-Val-Phe-Arg-Ser-Leu-Lys↓Tyr-Ala-Glu-Ser-Asp

(viii) hProBDNF (50-63)

Lys-Ala-Gly-Ser-Arg-Gly-Leu-Thr↓Ser-Leu-Ala-Asp-Thr-Phe

(ix) SREBP-2 27 mer

Gly-Gly-Ala-His-Asp-Ser-Asp-Gln-His-Pro-His-Ser-Gly-Ser-Gly-Arg-Ser-Val-Leu↓Ser-Phe-Glu-Ser-Gly-Ser-Gly-Gly

(x) SREBP-2 10 mer

Ser-Gly-Ser-Gly-Arg-Ser-Val-Leu↓Ser-Phe-Glu-Ser

These peptides were examined as possible substrates of SKI-1. Our data indicate that only the **peptides (iii), (iv), (vii), (viii) (ix) and (x)** are efficiently cleaved by the recombinant SKI-1.

NOVEL FLUOROGENIC SUBSTRATE BASED ASSAY OF SKI-1 ACTIVITY:

Based on the results reported above with various synthetic peptides we designed a number of internally quenched fluorogenic substrates of SKI-1. *Our main goal was to develop a rapid and a sensitive method for the assay of SKI-1 enzymatic activity.* SKI-1 activity was monitored by following the cleavage of suitable peptide substrates with HPLC that is often extremely slow and cumbersome. The following internally quenched fluorogenic peptides were synthesized and tested as substrates for SKI-1:

(a) QSKI (132-142):

Abz-Val-Phe-Arg-Ser-Leu-Lys ↓Tyr-Ala-Glu-Ser-Asp-Tyr(NO_2)-Ala

(b) QSKI (134-142):

Abz-Arg-Ser-Leu-Lys ↓ Tyr-Ala-Glu-Ser-Asp-Tyr(NO_2)-Ala

(c) QSKI (178-188)

Abz-Arg-His-Ser-Ser-Arg-Arg-Leu-Leu ↓ Arg-Ala-Ile-Tyr(NO_2)-Ala

(d) QSKI (181-187+Leu+Glu)

Abz-Ser-Arg-Arg-Leu-Leu ↓ Arg-Ala-Leu-Glu-Tyr(NO_2)-Ala

(e) QBDNF (47-58)

Abz-Asn-Gly-Pro-Lys-Ala-Gly-Ser-Arg-Gly-Leu-Thr ↓ Ser-Tyr(NO_2)-Ala

The main feature of these peptides is the incorporation of two special amino acids namely **Abz [Ortho amino benzoic acid also known as anthranalic acid]** and **Tyr(NO_2) [3-nitro Tyrosin]** at the amino (N-) and carboxy (C-) terminal end of the peptide chain respectively. **Abz, an electron donor,** is a powerful fluorescent moiety whereas **Tyr(NO_2), an electron acceptor,** acts as a fluorescence quench group. All the above peptides exhibit weak fluorescence background values (at λ_{ex} = **320 nm** and λ_{em} = **420 nm**). It is expected that upon cleavage by the proteolytic action of SKI, these peptides will release two peptide

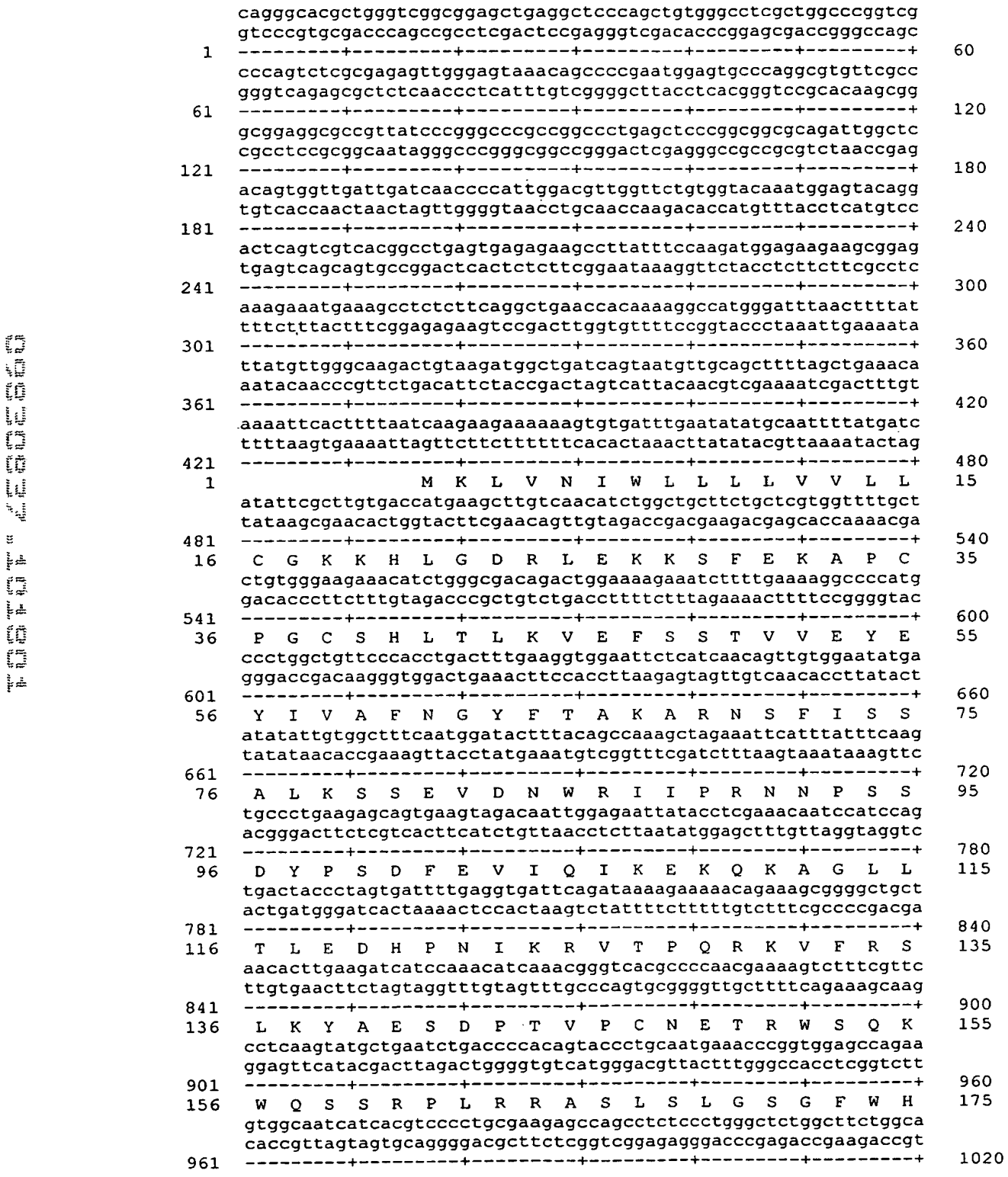
fragments of which the **Abz-containing N-terminal part** should display a very high degree of fluorescence. The net result will be the increase of fluorescence intensity that can be measured very accurately with a fluorimeter instrument. This technique of measurement of enzymatic activity has been applied to a number of enzymes { F. Jean, A. Boudreault, A. Basak, N. G. Seidah and C. Lazure.,., J. Biol. Chem., 1995, **270,** 19225-19231}

RESULTS

Our data indicates that among the above quenched fluorogenic peptides, **peptide (a)** is most effective as a substrate for SKI-1. In fact the measurement of kinetic parameters **(V_{max}/Km)** indicted that this peptide is **6-fold** more efficient that the nearest candidate **quenched peptide (b).** HPLC analysis using both UV and fluorescence detector systems clearly revealed a single site of cleavage in **peptides (a)** and **(b)** (as indicated above by a vertical arrow ↓), again reenforcing the notion that the preferred sequence motif for SKI-1 is characterized by the presence of an Arg residue at P4 , an alkyl hydrophobic residue at P2 and possibly an aromatic hydrophobic residue at P1'. ***Therefore, peptide (a) is a highly specific fluorogenic substrate for monitoring the activity of SKI-1***

This invention has been described in details hereinabove, and it will be readily apparent to the skilled artisan that modifications can be made thereto without departing form the teachings of the present disclosure. These modifications are considered within the scope of the present invention, as defined in the appended claims.

Human SKI-1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gtcccgtgcgacccagccgcctcgactccgagggtcgacacccggagcgaccgggccagc
1 60
cccagtctcgcgagagttgggagtaaacagccccgaatggagtgcccaggcgtgttcgcc
gggtcagagcgctctcaaccctcatttgtcggggcttacctcacgggtccgcacaagcgg
61 120
gcggaggcgccgttatcccgggcccgccggccctgagctcccggcggcgcagattggctc
cgcctccgcggcaatagggcccgggcggccgggactcgagggccgccgcgtctaaccgag
121 180
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tgtcaccaactaactagttggggtaacctgcaaccaagacaccatgtttacctcatgtcc
181 240
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301 360
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361 420
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421 480
M K L V N I W L L L L V V L L
1 15
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tataagcgaacactggtacttcgaacagttgtagaccgacgaagacgagcaccaaaacga
481 540
C G K K H L G D R L E K K S F E K A P C
16 35
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541 600
P G C S H L T L K V E F S S T V V E Y E
36 55
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Y I V A F N G Y F T A K A R N S F I S S
56 75
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721 780
D Y P S D F E V I Q I K E K Q K A G L L
96 115
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781 840
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116 135
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ttgtgaacttctagtaggtttgtagtttgcccagtgcggggttgcttttcagaaagcaag
841 900
L K Y A E S D P T V P C N E T R W S Q K
136 155
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901 960
W Q S S R P L R R A S L S L G S G F W H
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961 1020

```
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 196   T  L  Q  A  D  V  L  W  Q  M  G  Y  T  G  A  N  V  R  V  A    215
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1081   ---------+---------+---------+---------+---------+---------+  1140
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1141   ---------+---------+---------+---------+---------+---------+  1200
 236   N  W  T  N  E  R  T  L  D  D  G  L  G  H  G  T  F  V  A  G    255
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1201   ---------+---------+---------+---------+---------+---------+  1260
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1261   ---------+---------+---------+---------+---------+---------+  1320
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1321   ---------+---------+---------+---------+---------+---------+  1380
 296   A  I  L  K  K  I  D  V  L  N  L  S  I  G  G  P  D  F  M  D    315
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1381   ---------+---------+---------+---------+---------+---------+  1440
 316   H  P  F  V  D  K  V  W  E  L  T  A  N  N  V  I  M  V  S  A    335
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1561   ---------+---------+---------+---------+---------+---------+  1620
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1621   ---------+---------+---------+---------+---------+---------+  1680
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1681   ---------+---------+---------+---------+---------+---------+  1740
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```

```
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1921   ---------+---------+---------+---------+---------+---------+  1980
 496   Y  M  W  P  Y  C  S  Q  P  I  Y  Y  G  G  M  P  T  V  V  N    515
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1981   ---------+---------+---------+---------+---------+---------+  2040
 516   V  T  I  L  N  G  M  G  V  T  G  R  I  V  D  K  P  D  W  Q    535
       tgtcaccatcctcaacggcatgggagtcacaggaagaattgtagataagcctgactggca
       acagtggtaggagttgccgtaccctcagtgtccttcttaacatctattcggactgaccgt
2041   ---------+---------+---------+---------+---------+---------+  2100
 536   P  Y  L  P  Q  N  G  D  N  I  E  V  A  F  S  Y  S  S  V  L    555
       gccctatttgccacagaacggagacaacattgaagttgccttctcctactcctcggtctt
       cgggataaacggtgtcttgcctctgttgtaacttcaacggaagaggatgaggagccagaa
2101   ---------+---------+---------+---------+---------+---------+  2160
 556   W  P  W  S  G  Y  L  A  I  S  I  S  V  T  K  K  A  A  S  W    575
       atggccttggtcgggctacctggccatctccatttctgtgaccaagaaagcggcttcctg
       taccggaaccagcccgatggaccggtagaggtaaagacactggttctttcgccgaaggac
2161   ---------+---------+---------+---------+---------+---------+  2220
 576   E  G  I  A  Q  G  H  V  M  I  T  V  A  S  P  A  E  T  E  S    595
       ggaaggcattgctcagggccatgtcatgatcactgtggcttccccagcagagacagagtc
       ccttccgtaacgagtcccggtacagtactagtgacaccgaaggggtcgtctctgtctcag
2221   ---------+---------+---------+---------+---------+---------+  2280
 596   K  N  G  A  E  Q  T  S  T  V  K  L  P  I  K  V  K  I  I  P    615
       aaaaaatggtgcagaacagacttcaacagtaaagctccccattaaggtgaagataattcc
       ttttttaccacgtcttgtctgaagttgtcatttcgaggggtaattccacttctattaagg
2281   ---------+---------+---------+---------+---------+---------+  2340
 616   T  P  P  R  S  K  R  V  L  W  D  Q  Y  H  N  L  R  Y  P  P    635
       tactccccccgcgaagcaagagagttctctgggatcagtaccacaacctccgctatccacc
       atgaggggggcgcttcgttctctcaagagaccctagtcatggtgttggaggcgataggtgg
2341   ---------+---------+---------+---------+---------+---------+  2400
 636   G  Y  F  P  R  D  N  L  R  M  K  N  D  P  L  D  W  N  G  D    655
       tggctatttccccagggataatttaaggatgaagaatgacccttttagactggaatggtga
       accgataaaggggtccctattaaattcctacttcttactgggaaatctgaccttaccact
2401   ---------+---------+---------+---------+---------+---------+  2460
 656   H  I  H  T  N  F  R  D  M  Y  Q  H  L  R  S  M  G  Y  F  V    675
       tcacatccacaccaatttcagggatatgtaccagcatctgagaagcatgggctactttgt
       agtgtaggtgtggttaaagtccctatacatggtcgtagactcttcgtacccgatgaaaca
2461   ---------+---------+---------+---------+---------+---------+  2520
 676   E  V  L  G  A  P  F  T  C  F  D  A  S  Q  Y  G  T  L  L  M    695
       agaggtcctcggggccccccttcacgtgttttgatgccagtcagtatggcactttgctgat
       tctccaggagccccgggggaagtgcacaaaactacggtcagtcataccgtgaaacgacta
2521   ---------+---------+---------+---------+---------+---------+  2580
 696   V  D  S  E  E  E  Y  F  P  E  E  I  A  K  L  R  R  D  V  D    715
       ggtggacagtgaggaggagtacttccctgaagagatcgccaagctccggagggacgtgga
       ccacctgtcactcctcctcatgaagggacttctctagcggttcgaggcctccctgcacct
2581   ---------+---------+---------+---------+---------+---------+  2640
 716   N  G  L  S  L  V  I  F  S  D  W  Y  N  T  S  V  M  R  K  V    735
       caacggcctctcgctcgtcatcttcagtgactggtacaacacttctgttatgagaaaagt
       gttgccggagagcgagcagtagaagtcactgaccatgttgtgaagacaatactcttttca
2641   ---------+---------+---------+---------+---------+---------+  2700
 736   K  F  Y  D  E  N  T  R  Q  W  W  M  P  D  T  G  G  A  N  I    755
       gaagttttatgatgaaaacacaaggcagtggtggatgccggataccggaggagctaacat
       cttcaaaatactacttttgtgttccgtcaccacctacggcctatggcctcctcgattgta
2701   ---------+---------+---------+---------+---------+---------+  2760
 756   P  A  L  N  E  L  L  S  V  W  N  M  G  F  S  D  G  L  Y  E    775
       cccagctctgaatgagctgctgtctgtgtggaacatggggttcagcgatggcctgtatga
       gggtcgagacttactcgacgacagacacaccttgtaccccaagtcgctaccggacatact
2761   ---------+---------+---------+---------+---------+---------+  2820
```

```
 776   G  E  F  T  L  A  N  H  D  M  Y  Y  A  S  G  C  S  I  A  K    795
       aggggagttcaccctggccaaccatgacatgtattatgcgtcagggtgcagcatcgcgaa
       tcccctcaagtgggaccggttggtactgtacataatacgcagtcccacgtcgtagcgctt
2821   ---------+---------+---------+---------+---------+---------+  2880
 796   F  P  E  D  G  V  V  I  T  Q  T  F  K  D  Q  G  L  E  V  L    815
       gtttccagaagatggcgtcgtgataacacagactttcaaggaccaaggattggaggtttt
       caaaggtcttctaccgcagcactattgtgtctgaaagttcctggttcctaacctccaaaa
2881   ---------+---------+---------+---------+---------+---------+  2940
 816   K  Q  E  T  A  V  V  E  N  V  P  I  L  G  L  Y  Q  I  P  A    835
       aaagcaggaaacagcagttgttgaaaacgtccccattttgggactttatcagattccagc
       tttcgtcctttgtcgtcaacaacttttgcaggggtaaaaccctgaaatagtctaaggtcg
2941   ---------+---------+---------+---------+---------+---------+  3000
 836   E  G  G  G  R  I  V  L  Y  G  D  S  N  C  L  D  D  S  H  R    855
       tgagggtggaggccggattgtactgtatggggactccaattgcttggatgacagtcaccg
       actcccacctccggcctaacatgacataccccctgaggttaacgaacctactgtcagtggc
3001   ---------+---------+---------+---------+---------+---------+  3060
 856   Q  K  D  C  F  W  L  L  D  A  L  L  Q  Y  T  S  Y  G  V  T    875
       acagaaggactgcttttggcttctggatgccctcctccagtacacatcgtatggggtgac
       tgtcttcctgacgaaaaccgaagacctacgggaggaggtcatgtgtagcataccccactg
3061   ---------+---------+---------+---------+---------+---------+  3120
 876   P  P  S  L  S  H  S  G  N  R  Q  R  P  P  S  G  A  G  S  V    895
       accgcctagcctcagtcactctgggaaccgccagcgccctcccagtggagcaggctcagt
       tggcggatcggagtcagtgagacccttggcggtcgcgggagggtcacctcgtccgagtca
3121   ---------+---------+---------+---------+---------+---------+  3180
 896   T  P  E  R  M  E  G  N  H  L  H  R  Y  S  K  V  L  E  A  H    915
       cactccagagaggatggaaggaaaccatcttcatcggtactccaaggttctggaggccca
       gtgaggtctctcctaccttcctttggtagaagtagccatgaggttccaagacctccgggt
3181   ---------+---------+---------+---------+---------+---------+  3240
 916   L  G  D  P  K  P  R  P  L  P  A  C  P  R  L  S  W  A  K  P    935
       tttgggagacccaaaacctcggcctctaccagcctgtccacgcttgtcttgggccaagcc
       aaaccctctgggttttggagccggagatggtcggacaggtgcgaacagaacccggttcgg
3241   ---------+---------+---------+---------+---------+---------+  3300
 936   Q  P  L  N  E  T  A  P  S  N  L  W  K  H  Q  K  L  L  S  I    955
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       tgtcggaaatttgctctgccgcgggtcattggaaacctttgtagtcttcgatgagaggta
3301   ---------+---------+---------+---------+---------+---------+  3360
 956   D  L  D  K  V  V  L  P  N  F  R  S  N  R  P  Q  V  R  P  L    975
       tgacctggacaaggtggtgttacccaactttcgatcgaatcgccctcaagtgaggccctt
       actggacctgttccaccacaatgggttgaaagctagcttagcgggagttcactccgggaa
3361   ---------+---------+---------+---------+---------+---------+  3420
 976   S  P  G  E  S  G  A  W  D  I  P  G  G  I  M  P  G  R  Y  N    995
       gtcccctggagagagcggcgcctgggacattcctggagggatcatgcctggccgctacaa
       caggggacctctctcgccgcggaccctgtaaggacctccctagtacggaccggcgatgtt
3421   ---------+---------+---------+---------+---------+---------+  3480
 996   Q  E  V  G  Q  T  I  P  V  F  A  F  L  G  A  M  V  V  L  A   1015
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       ggtcctccacccggtctggtaaggacagaaacggaaggaccctcggtaccaccaggaccg
3481   ---------+---------+---------+---------+---------+---------+  3540
1016   F  F  V  V  Q  I  N  K  A  K  S  R  P  K  R  R  K  P  R  V   1035
       cttctttgtggtacaaatcaacaaggccaagagcaggccgaagcggaggaagcccagggt
       gaagaaacaccatgtttagttgttccggttctcgtccggcttcgcctccttcgggtccca
3541   ---------+---------+---------+---------+---------+---------+  3600
1036   K  R  P  Q  L  M  Q  Q  V  H  P  P  K  T  P  S  V  *.        1053
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3601   ---------+---------+---------+---------+---------+---------+  3660
       agcctggctgaccgtgagggccagagagagccttcacggacggcgctggtgggtgagccg
       tcggaccgactggcactcccggtctctctcggaagtgcctgccgcgaccacccactcggc
3661   ---------+---------+---------+---------+---------+---------+  3720
       agctgtggtggcggctggtttaaaagggatccagtttccagctgcaggtttgttagagtc
       tcgacaccaccgccgaccaaattttccctaggtcaaaggtcgacgtccaaacaatctcag
3721   ---------+---------+---------+---------+---------+---------+  3780
```

```
      tgttctacatgggcctgccctcctgtgatgggcagaggctcctggtacatcgagaagatt
      acaagatgtacccggacgggaggacactacccgtctccgaggaccatgtagctcttctaa
3781  ---------+---------+---------+---------+---------+---------+  3840
      cctgtggatcccgtcaggagggacttagtggctctgccgccagtgagacttcccgccggc
      ggacacctagggcagtcctccctgaatcaccgagacggcggtcactctgaagggcggccg
3841  ---------+---------+---------+---------+---------+---------+  3900
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3901  ---------+---------+---------+---------+---------+---------+  3960
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      ccccttcctacatgaaaggtttgtttactatgttgggactggttcgattttctgcgaaca
3961  ---------+---------+---------+---------+---------+---------+  4020
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      atttccgataaaagatataaataacaacccttttcagtgaaatttctgaacacgataaac
4021  ---------+---------+---------+---------+---------+---------+  4080
      gaagcaaagctatttttttgtcagtggaatgcagtttttttactattccatcatgagga
      cttcgtttcgataaaaaaaaacagtcaccttacgtcaaaaaaatgataaggtagtactcct
4081  ---------+---------+---------+---------+---------+---------+  4140
      acaacatagattccatgatctttttaatgacagtacagactgagatttgaaggaaacatg
      tgttgtatctaaggtactagaaaaattactgtcatgtctgactctaaacttcctttgtac
4141  ---------+---------+---------+---------+---------+---------+  4200
      cacaaatctgtaaaacatagaccttcgctttatttttgtaagtatcacctgccaccatgt
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4201  ---------+---------+---------+---------+---------+---------+  4260
      tttgtaatttgaggtcttgatttcaccattgtcggtgaagaaaattttcaataaatatgt
      aaacattaaactccagaactaaagtggtaacagccacttcttttaaaagttatttataca
4261  ---------+---------+---------+---------+---------+---------+  4320
      attacccgtctgaagctt
      taatgggcagacttcgaa
4321  ---------+--------  4338
```

Rat SKI-1

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   1  GCGAGTAAACATCCCCCGAATGGATACCCGAGGCGTGTTCGCGGCGGAGGCCCCGTTTTC   60
      CGCTCATTTGTAGGGGGCTTACCTATGGGCTCCGCACAAGCGCCGCCTCCGGGGCAAAAG

  61  CCGGGTCCGCCGATCCCGAGCCTGAGGCGACGCAGATCGGCTCAGAGCGGTGGCTTGGGC   120
      GGCCCAGGCGGCTAGGGCTCGGACTCCGCTGCGTCTAGCCGAGTCTCGCCACCGAACCCG

 121  TCCTGCTAGATTTGGGTCTGTGGTACAAATGGAGTTTAGGACTCAGTGGACTCGGCCCTA   180
      AGGACGATCTAAACCCAGACACCATGTTTACCTCAAATCCTGAGTCACCTGAGCCGGGAT

 181  ATGAGAGAAGCCCCCTGTCCAAGATGGAGAAGAAGCGGAGAAAGAAATGAAAGCCTCTTT   240
      TACTCTCTTCGGGGGACAGGTTCTACCTCTTCTTCGCCTCTTTCTTTACTTTCGGAGAAA

 241  TTGGGCCAAGCTGTGGGTGACCATGGGACTGAGGTTTTCTTTACGTTGGACAAGTCTGTA   300
      AACCCGGTTCGACACCCACTGGTACCCTGACTCCAAAAGAAATGCAACCTGTTCAGACAT

 301  GGATGGCTGATCAGTAAGGTTGCAGCTTTTAGCGAAAACAGAAATCCACTTCTGATCAAG   360
      CCTACCGACTAGTCATTCCAACGTCGAAAATCGCTTTTGTCTTTAGGTGAAGACTAGTTC

   1                                                          M     1
 361  GAAGAGCCTAGTGCAATTTGAATTTATGCAATTTTATGACCATATTCACTTAGGACCATG   420
      CTTCTCGGATCACGTTAAACTTAAATACGTTAAAATACTGGTATAAGTGAATCCTGGTAC

   2  K  L  V  N  I  W  L  L  L  L  V  V  L  L  C  G  K  K  H  L     21
 421  AAGCTCGTCAACATCTGGCTTCTTCTGCTGGTGGTTTTGCTCTGTGGGAAAAAGCATCTG   480
      TTCGAGCAGTTGTAGACCGAAGAAGACGACCACCAAAACGAGACACCCTTTTTCGTAGAC

  22  G  D  R  L  G  K  K  A  F  E  K  A  P  C  P  S  C  S  H  L     41
 481  GGTGACAGGCTGGGGAAGAAAGCTTTTGAAAAGGCCCCATGCCCCAGCTGTTCCCACCTG   540
      CCACTGTCCGACCCCTTCTTTCGAAAACTTTTCCGGGGTACGGGGTCGACAAGGGTGGAC

  42  T  L  K  V  E  F  S  S  T  V  V  E  Y  E  Y  I  V  A  F  N     61
 541  ACTTTGAAGGTGGAATTCTCCTCAACTGTGGTGGAATATGAATATATTGTGGCTTTCAAC   600
      TGAAACTTCCACCTTAAGAGGAGTTGACACCACCTTATACTTATATAACACCGAAAGTTG

  62  G  Y  F  T  A  K  A  R  N  S  F  I  S  S  A  L  K  S  S  E     81
 601  GGATACTTCACAGCCAAAGCTAGAAACTCATTTATTTCAAGTGCTCTAAAAAGCAGTGAA   660
      CCTATGAAGTGTCGGTTTCGATCTTTGAGTAAATAAAGTTCACGAGATTTTTCGTCACTT

  82  V  D  N  W  R  I  I  P  R  N  N  P  S  S  D  Y  P  S  D  F    101
 661  GTGGACAACTGGAGAATAATACCTCGGAACAACCCATCTAGTGACTACCCTAGTGATTTT   720
      CACCTGTTGACCTCTTATTATGGAGCCTTGTTGGGTAGATCACTGATGGGATCACTAAAA

 102  E  V  I  Q  I  K  E  K  Q  K  A  G  L  L  T  L  E  D  H  P    121
 721  GAGGTGATTCAGATAAAAGAGAAGCAGAAGGCGGGGCTGCTCACACTTGAAGATCACCCA   780
      CTCCACTAAGTCTATTTTCTCTTCGTCTTCCGCCCCGACGAGTGTGAACTTCTAGTGGGT
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SUBSTITUTE SHEET (RULE 26)

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122  N  I  K  R  V  T  P  Q  R  K  V  F  R  S  L  K  F  A  E  S    141
781  AACATCAAGCGGGTGACACCCCAGCGGAAAGTCTTTCGTTCCCTGAAGTTTGCTGAATCC  840
     TTGTAGTTCGCCCACTGTGGGGTCGCCTTTCAGAAAGCAAGGGACTTCAAACGACTTAGG

142  D  P  I  V  P  C  N  E  T  R  W  S  Q  K  W  Q  S  S  R  P    161
841  GACCCCATTGTGCCCTGTAATGAGACCCGGTGGAGCCAGAAGTGGCAGTCATCACGTCCC  900
     CTGGGGTAACACGGGACATTACTCTGGGCCACCTCGGTCTTCACCGTCAGTAGTGCAGGG

162  L  K  R  A  S  L  S  L  G  S  G  F  W  H  A  T  G  R  H  S    181
901  CTGAAAAGAGCCAGTCTCTCCCTGGGCTCTGGATTCTGGCATGCAACAGGAAGGCATTCA  960
     GACTTTTCTCGGTCAGAGAGGGACCCGAGACCTAAGACCGTACGTTGTCCTTCCGTAAGT

182  S  R  R  L  L  R  A  I  P  R  Q  V  A  Q  T  L  Q  A  D  V    201
961  AGTCGACGATTGCTGAGAGCCATTCCTCGCCAGGTTGCCCAGACATTGCAGGCAGATGTG  1020
     TCAGCTGCTAACGACTCTCGGTAAGGAGCGGTCCAACGGGTCTGTAACGTCCGTCTACAC

202  L  W  Q  M  G  Y  T  G  A  N  V  R  V  A  V  F  D  T  G  L    221
1021 CTTTGGCAGATGGGATACACAGGTGCTAATGTCAGGGTTGCCGTTTTTGATACTGGGCTC  1080
     GAAACCGTCTACCCTATGTGTCCACGATTACAGTCCCAACGGCAAAAACTATGACCCGAG

222  S  E  K  H  P  H  F  K  N  V  K  E  R  T  N  W  T  N  E  R    241
1081 AGTGAGAAGCATCCACATTTCAAGAATGTGAAGGAAAGAACCAACTGGACCAATGAGCGG  1140
     TCACTCTTCGTAGGTGTAAAGTTCTTACACTTCCTTTCTTGGTTGACCTGGTTACTCGCC

242  T  L  D  D  G  L  G  H  G  T  F  V  A  G  V  I  A  S  M  R.   261
1141 ACCCTGGACGATGGGCTGGGCCATGGCACATTCGTTGCAGGTGTGATTGCCAGCATGAGA  1200
     TGGGACCTGCTACCCGACCCGGTACCGTGTAAGCAACGTCCACACTAACGGTCGTACTCT

262  E  C  Q  G  F  A  P  D  A  E  L  H  I  F  R  V  F  T  N  N    281
1201 GAGTGCCAAGGATTTGCCCCAGATGCAGAGCTGCACATCTTCAGGGTCTTTACCAACAAT  1260
     CTCACGGTTCCTAAACGGGGTCTACGTCTCGACGTGTAGAAGTCCCAGAAATGGTTGTTA

282  Q  V  S  Y  T  S  W  F  L  D  A  F  N  Y  A  I  L  K  K  M    301
1261 CAGGTGTCTTACACGTCTTGGTTTTTGGATGCCTTCAACTATGCCATCCTAAAGAAGATG  1320
     GTCCACAGAATGTGCAGAACCAAAAACCTACGGAAGTTGATACGGTAGGATTTCTTCTAC

302  D  V  L  N  L  S  I  G  G  P  D  F  M  D  H  P  F  V  D  K    321
1321 GACGTTCTGAACCTTAGCATCGGTGGGCCTGACTTCATGGATCACCCCTTTGTTGACAAG  1380
     CTGCAAGACTTGGAATCGTAGCCACCCGGACTGAAGTACCTAGTGGGGAAACAACTGTTC

322  V  W  E  L  T  A  N  N  V  I  M  V  S  A  I  G  N  D  G  P    341
1381 GTATGGGAATTAACAGCGAACAATGTAATCATGGTTTCTGCTATTGGCAATGATGGACCT  1440
     CATACCCTTAATTGTCGCTTGTTACATTAGTACCAAAGACGATAACCGTTACTACCTGGA

342  L  Y  G  T  L  N  N  P  A  D  Q  M  D  V  I  G  V  G  G  I    361
1441 CTCTATGGCACTCTGAATAACCCTGCTGATCAGATGGATGTGATTGGAGTGGGTGGCATT  1500
     GAGATACCGTGAGACTTATTGGGACGACTAGTCTACCTACACTAACCTCACCCACCGTAA

362  D  F  E  D  N  I  A  R  F  S  S  R  G  M  T  T  W  E  L  P    381
1501 GACTTTGAAGACAACATCGCCCGCTTCTCTTCCAGGGGAATGACTACCTGGGAACTACCG  1560
     CTGAAACTTCTGTTGTAGCGGGCGAAGAGAAGGTCCCCTTACTGATGGACCCTTGATGGC
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 382  G  G  Y  G  R  V  K  P  D  I  V  T  Y  G  A  G  V  R  G  S    401
1561  GGAGGCTATGGTCGTGTGAAGCCTGACATTGTCACCTATGGTGCTGGAGTGCGGGGTTCT  1620
      CCTCCGATACCAGCACACTTCGGACTGTAACAGTGGATACCACGACCTCACGCCCCAAGA

 402  G  V  K  G  G  C  R  A  L  S  G  T  S  V  A  S  P  V  V  A    421
1621  GGTGTGAAAGGGGGCTGCCGTGCACTCTCAGGGACCAGTGTCGCCTCCCCAGTGGTTGCT  1680
      CCACACTTTCCCCCGACGGCACGTGAGAGTCCCTGGTCACAGCGGAGGGGTCACCAACGA

 422  G  A  V  T  L  L  V  S  T  V  Q  K  R  E  L  V  N  P  A  S    441
1681  GGGGCTGTCACCTTGTTAGTAAGCACAGTACAGAAGCGGGAGCTAGTGAATCCTGCCAGT  1740
      CCCCGACAGTGGAACAATCATTCGTGTCATGTCTTCGCCCTCGATCACTTAGGACGGTCA

 442  V  K  Q  A  L  I  A  S  A  R  R  L  P  G  V  N  M  F  E  Q    461
1741  GTGAAGCAAGCTTTGATAGCATCAGCCCGGAGACTTCCTGGTGTCAACATGTTTGAGCAA  1800
      CACTTCGTTCGAAACTATCGTAGTCGGGCCTCTGAAGGACCACAGTTGTACAAACTCGTT

 462  G  H  G  K  L  D  L  L  R  A  Y  Q  I  L  S  S  Y  K  P  Q    481
1801  GGCCATGGCAAGTTGGATCTACTGCGAGCCTATCAGATCCTCAGCAGCTATAAACCGCAG  1860
      CCGGTACCGTTCAACCTAGATGACGCTCGGATAGTCTAGGAGTCGTCGATATTTGGCGTC

 482  A  S  L  S  P  S  Y  I  D  L  T  E  C  P  Y  M  W  P  Y  C    501
1861  GCGAGCCTGAGTCCTAGCTACATCGACCTGACTGAGTGTCCCTACATGTGGCCCTACTGC  1920
      CGCTCGGACTCAGGATCGATGTAGCTGGACTGACTCACAGGGATGTACACCGGGATGACG

 502  S  Q  P  I  Y  Y  G  G  M  P  T  I  V  N  V  T  I  L  N  G    521
1921  TCCCAGCCCATCTACTATGGAGGAATGCCAACAATTGTTAATGTCACCATCCTCAATGGC  1980
      AGGGTCGGGTAGATGATACCTCCTTACGGTTGTTAACAATTACAGTGGTAGGAGTTACCG

 522  M  G  V  T  G  R  I  V  D  K  P  E  W  R  P  Y  L  P  Q  N    541
1981  ATGGGAGTTACAGGAAGAATTGTGGATAAGCCTGAGTGGCGACCCTATTTACCACAGAAT  2040
      TACCCTCAATGTCCTTCTTAACACCTATTCGGACTCACCGCTGGGATAAATGGTGTCTTA

 542  G  D  N  I  E  V  A  F  S  Y  S  S  V  L  W  P  W  S  G  Y    561
2041  GGAGACAACATTGAAGTGGCCTTCTCCTACTCCTCAGTGTTGTGGCCTTGGTCAGGTTAC  2100
      CCTCTGTTGTAACTTCACCGGAAGAGGATGAGGAGTCACAACACCGGAACCAGTCCAATG

 562  L  A  I  S  I  S  V  T  K  K  A  A  S  W  E  G  I  A  Q  G    581
2101  CTTGCCATCTCCATTTCTGTGACCAAGAAGGCAGCTTCCTGGGAAGGCATCGCGCAGGGC  2160
      GAACGGTAGAGGTAAAGACACTGGTTCTTCCGTCGAAGGACCCTTCCGTAGCGCGTCCCG

 582  H  I  M  I  T  V  A  S  P  A  E  T  E  L  K  N  G  A  E  H    601
2161  CACATCATGATCACAGTGGCTTCCCCAGCAGAGACGGAATTAAAAAATGGTGCCGAGCAT  2220
      GTGTAGTACTAGTGTCACCGAAGGGGTCGTCTCTGCCTTAATTTTTTACCACGGCTCGTA

 602  T  S  T  V  K  L  P  I  K  V  K  I  I  P  T  P  P  R  S  K    621
2221  ACTTCCACAGTGAAGCTGCCCATCAAGGTGAAGATCATTCCCACCCCTCCTCGGAGCAAG  2280
      TGAAGGTGTCACTTCGACGGGTAGTTCCACTTCTAGTAAGGGTGGGGAGGAGCCTCGTTC
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SUBSTITUTE SHEET (RULE 26)

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 622  R  V  L  W  D  Q  Y  H  N  L  R  Y  P  P  G  Y  F  P  R  D    641
2281  AGAGTCCTCTGGGACCAGTACCACAACCTCCGCTACCCACCCGGCTACTTCCCCAGGGAC  2340
      TCTCAGGAGACCCTGGTCATGGTGTTGGAGGCGATGGGTGGGCCGATGAAGGGGTCCCTG

 642  N  L  R  M  K  N  D  P  L  D  W  N  G  D  H  V  H  T  N  F    661
2341  AACTTGCGGATGAAGAATGATCCTTTAGACTGGAATGGCGACCACGTCCACACCAACTTC  2400
      TTGAACGCCTACTTCTTACTAGGAAATCTGACCTTACCGCTGGTGCAGGTGTGGTTGAAG

 662  R  D  M  Y  Q  H  L  R  S  M  G  Y  F  V  E  V  L  G  A  P    681
2401  AGGGACATGTACCAGCATCTGCGCAGCATGGGCTACTTTGTGGAGGTGCTTGGTGCCCCA  2460
      TCCCTGTACATGGTCGTAGACGCGTCGTACCCGATGAAACACCTCCACGAACCACGGGGT

 682  F  T  C  F  D  A  T  Q  Y  G  T  L  L  M  V  D  S  E  E  E    701
2461  TTCACATGCTTTGACGCCACGCAGTACGGCACTCTGCTTATGGTGGACAGTGAGGAAGAG  2520
      AAGTGTACGAAACTGCGGTGCGTCATGCCGTGAGACGAATACCACCTGTCACTCCTTCTC

 702  Y  F  P  E  E  I  A  K  L  R  R  D  V  D  N  G  L  S  L  V    721
2521  TACTTCCCTGAGGAGATTGCTAAGCTGAGGAGGGACGTGGACAATGGCCTTTCCCTTGTC  2580
      ATGAAGGGACTCCTCTAACGATTCGACTCCTCCCTGCACCTGTTACCGGAAAGGGAACAG

 722  V  F  S  D  W  Y  N  T  S  V  M  R  K  V  K  F  Y  D  E  N    741
2581  GTCTTCAGTGACTGGTACAACACTTCTGTTATGAGAAAAGTGAAGTTTTACGATGAAAAC  2640
      CAGAAGTCACTGACCATGTTGTGAAGACAATACTCTTTTCACTTCAAAATGCTACTTTTG

 742  T  R  Q  W  W  M  P  D  T  G  G  A  N  V  P  A  L  N  E  L    761
2641  ACAAGGCAGTGGTGGATGCCAGATACTGGAGGAGCCAACGTCCCAGCTCTAAACGAGCTG  2700
      TGTTCCGTCACCACCTACGGTCTATGACCTCCTCGGTTGCAGGGTCGAGATTTGCTCGAC

 762  L  S  V  W  N  M  G  F  S  D  G  L  Y  E  G  E  F  A  L  A    781
2701  CTGTCTGTGTGGAACATGGGGTTCAGTGACGGCCTGTATGAAGGGGAGTTTGCCCTGGCA  2760
      GACAGACACACCTTGTACCCCAAGTCACTGCCGGACATACTTCCCCTCAAACGGGACCGT

 782  N  H  D  M  Y  Y  A  S  G  C  S  I  A  R  F  P  E  D  G  V    801
2761  AACCACGACATGTACTATGCATCGGGGTGCAGCATTGCCAGGTTTCCAGAAGATGGTGTG  2820
      TTGGTGCTGTACATGATACGTAGCCCCACGTCGTAACGGTCCAAAGGTCTTCTACCACAC

 802  V  I  T  Q  T  F  K  D  Q  G  L  E  V  L  K  Q  E  T  A  V    821
2821  GTGATCACACAGACTTTCAAGGACCAAGGATTGGAAGTCTTAAAACAAGAGACAGCAGTT  2880
      CACTAGTGTGTCTGAAAGTTCCTGGTTCCTAACCTTCAGAATTTTGTTCTCTGTCGTCAA

 822  V  D  N  V  P  I  L  G  L  Y  Q  I  P  A  E  G  G  G  R  I    841
2881  GTCGACAATGTCCCCATTCTGGGGCTATATCAGATTCCAGCTGAAGGTGGAGGCCGGATT  2940
      CAGCTGTTACAGGGGTAAGACCCCGATATAGTCTAAGGTCGACTTCCACCTCCGGCCTAA

 842  V  L  Y  G  D  S  N  C  L  D  D  S  H  R  Q  K  D  C  F  W    861
2941  GTGCTGTATGGAGACTCCAACTGCTTGGATGACAGTCACAGACAGAAGGACTGCTTTTGG  3000
      CACGACATACCTCTGAGGTTGACGAACCTACTGTCAGTGTCTGTCTTCCTGACGAAAACC
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SUBSTITUTE SHEET (RULE 26)

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 862  L  L  D  A  L  L  Q  Y  T  S  Y  G  V  T  P  P  S  L  S  H     881
3001  CTTCTGGATGCACTCCTTCAGTACACATCCTATGGTGTGACCCCTCCCAGCCTCAGCCAT  3060
      GAAGACCTACGTGAGGAAGTCATGTGTAGGATACCACACTGGGGAGGGTCGGAGTCGGTA

 882  S  G  N  R  Q  R  P  P  S  G  A  G  L  A  P  P  E  R  M  E     901
3061  TCAGGGAACCGGCAGCGCCCACCCAGCGGGGCTGGCTTGGCCCCTCCTGAAAGGATGGAA  3120
      AGTCCCTTGGCCGTCGCGGGTGGGTCGCCCCGACCGAACCGGGGAGGACTTTCCTACCTT

 902  G  N  H  L  H  R  Y  S  K  V  L  E  A  H  L  G  D  P  K  P     921
3121  GGAAACCACCTTCATCGCTACTCCAAAGTTCTTGAGGCCCACTTGGGAGACCCGAAACCT  3180
      CCTTTGGTGGAAGTAGCGATGAGGTTTCAAGAACTCCGGGTGAACCCTCTGGGCTTTGGA

 922  R  P  L  P  A  C  P  H  L  S  W  A  K  P  Q  P  L  N  E  T     941
3181  CGGCCCCTTCCAGCCTGTCCACACTTGTCGTGGGCCAAGCCACAGCCTTTGAATGAGACG  3240
      GCCGGGGAAGGTCGGACAGGTGTGAACAGCACCCGGTTCGGTGTCGGAAACTTACTCTGC

 942  A  P  S  N  L  W  K  H  Q  K  L  L  S  I  D  L  D  K  V  V     961
3241  GCACCCAGTAATCTTTGGAAACACCAGAAGCTGCTCTCCATTGACCTGGACAAAGTAGTG  3300
      CGTGGGTCATTAGAAACCTTTGTGGTCTTCGACGAGAGGTAACTGGACCTGTTTCATCAC

 962  L  P  N  F  R  S  N  R  P  Q  V  R  P  L  S  P  G  E  S  G     981
3301  TTACCCAACTTTCGCTCAAATCGCCCTCAAGTGAGACCTTTGTCCCCTGGAGAAAGTGGT  3360
      AATGGGTTGAAAGCGAGTTTAGCGGGAGTTCACTCTGGAAACAGGGGACCTCTTTCACCA

 982  A  W  D  I  P  G  G  I  M  P  G  R  Y  N  Q  E  V  G  Q  T    1001
3361  GCCTGGGACATTCCTGGAGGGATCATGCCTGGCCGCTACAACCAGGAAGTAGGCCAGACC  3420
      CGGACCCTGTAAGGACCTCCCTAGTACGGACCGGCGATGTTGGTCCTTCATCCGGTCTGG

1002  I  P  V  F  A  F  L  G  A  M  V  A  L  A  F  F  V  V  Q  I    1021
3421  ATCCCTGTTTTTGCCTTCCTTGGAGCCATGGTGGCCCTGGCCTTCTTCGTGGTACAGATC  3480
      TAGGGACAAAAACGGAAGGAACCTCGGTACCACCGGGACCGGAAGAAGCACCATGTCTAG

1022  S  K  A  K  S  R  P  K  R  R  R  P  R  A  K  R  P  Q  L  A    1041
3481  AGTAAGGCCAAGAGCCGGCCGAAGCGGAGGAGGCCCAGGGCAAAGCGTCCACAACTTGCA  3540
      TCATTCCGGTTCTCGGCCGGCTTCGCCTCCTCCGGGTCCCGTTTCGCAGGTGTTGAACGT

1042  Q  Q  A  H  P  A  R  T  P  S  V                                1052
3541  CAGCAGGCCCACCCTGCAAGGACCCCGTCAGTGTGATCATCACAGTGGCCAGACACAGAA  3600
      GTCGTCCGGGTGGGACGTTCCTGGGGCAGTCACACTAGTAGTGTCACCGGTCTGTGTCTT

3601  GCTGACAAGCTTTGAACCCCTCTGGTGGCCACACAGCATCAGAGAGCATCCTGGGAAGTG  3660
      CGACTGTTCGAAACTTGGGGAGACCACCGGTGTGTCGTAGTCTCTCGTAGGACCCTTCAC

3661  CCTGTTTCCAAGGAGCCCTATCTCTGGATTGTGGCTGGCTTAGTGTGTTCTGCCCAGACG  3720
      GGACAAAGGTTCCTCGGGATAGAGACCTAACACCGACCGAATCACACAAGACGGGTCTGC

3721  TCTATGAGGTACATCCTGCAGTGCCTCACTGTGTTTGGCTCTGGCCGAAGGTGCCCAGTA  3780
      AGATACTCCATGTAGGACGTCACGGAGTGACACAAACCGAGACCGGCTTCCACGGGTCAT
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SUBSTITUTE SHEET (RULE 26)

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3781  GCTCAGCCTCCGGTGGCATCAGGCCCAGTGACAGTGCACCAAAGACACAGAGCCTGGAAG   3840
      CGAGTCGGAGGCCACCGTAGTCCGGGTCACTGTCACGTGGTTTCTGTGTCTCGGACCTTC

3841  GGCTGTCGGGACATACTTTCTACATAATGCTACAACCCTGACCAAGCGAAGACAT   3895
      CCGACAGCCCTGTATGAAAGATGTATTACGATGTTGGGACTGGTTCGCTTCTGTA
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Mouse SKI-1

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  1    .    M  K  L  V  S  T  W  L  L  V  L  V  V  L  L  C  G  K   18
       GCATTCCATGAAGCTCGTCAGCACCTGGCTTCTTGTGCTGGTGGTTTTGCTCTGTGGGAA
  1    CGTAAGGTACTTCGAGCAGTCGTGGACCGAAGAACACGACCACCAAAACGAGACACCCTT   60

 19    R  H  L  G  D  R  L  G  T  R  A  L  E  K  A  P  C  P  S  C   38
       ACGGCACCTGGGCGACAGGCTGGGGACGAGAGCTTTGGAAAAGGCCCCGTGCCCCAGCTG
 61    TGCCGTGGACCCGCTGTCCGACCCCTGCTCTCGAAACCTTTTCCGGGGCACGGGGTCGAC  120

 39    S  H  L  T  L  K  V  E  F  S  S  T  V  V  E  Y  E  Y  I  V   58
       CTCCCACCTGACTTTGAAGGTGGAATTCTCTTCAACTGTGGTGGAGTACGAATATATTGT
121    GAGGGTGGACTGAAACTTCCACCTTAAGAGAAGTTGACACCACCTCATGCTTATATAACA  180

 59    A  F  N  G  Y  F  T  A  K  A  R  N  S  F  I  S  S  A  L  K   78
       GGCTTTCAACGGATACTTCACAGCCAAAGCTAGAAACTCATTTATTTCAAGTGCGCTGAA
181    CCGAAAGTTGCCTATGAAGTGTCGGTTTCGATCTTTGAGTAAATAAAGTTCACGCGACTT  240

 79    S  S  E  V  E  N  W  R  I  I  P  R  N  N  P  S  S  D  Y  P   98
       AAGCAGTGAAGTGGAAAACTGGAGAATAATACCTCGGAACAACCCATCCAGTGACTACCC
241    TTCGTCACTTCACCTTTTGACCTCTTATTATGGAGCCTTGTTGGGTAGGTCACTGATGGG  300

 99    S  D  F  E  V  I  Q  I  K  E  K  Q  K  A  G  L  L  T  L  E  118
       TAGTGATTTTGAGGTGATTCAGATAAAAGAGAAGCAGAAGGCGGGGCTGCTCACACTTGA
301    ATCACTAAAACTCCACTAAGTCTATTTTCTCTTCGTCTTCCGCCCCGACGAGTGTGAACT  360

119    D  H  P  N  I  K  R  V  T  P  Q  R  K  V  F  R  S  L  K  F  138
       AGATCACCCCAACATCAAGCGGGTGACACCCCAGCGGAAAGTCTTTCGTTCCCTCAAGTT
361    TCTAGTGGGGTTGTAGTTCGCCCACTGTGGGGTCGCCTTTCAGAAAGCAAGGGAGTTCAA  420

139    A  E  S  N  P  I  V  P  C  N  E  T  R  W  S  Q  K  W  Q  S  158
       TGCTGAATCCAACCCCATCGTGCCCTGTAATGAAACCCGGTGGAGCCAGAAGTGGCAGTC
421    ACGACTTAGGTTGGGGTAGCACGGGACATTACTTTGGGCCACCTCGGTCTTCACCGTCAG  480

159    S  R  P  L  K  R  A  S  L  S  L  G  S  G  F  W  H  A  T  G  178
       ATCACGTCCCCTGAAAAGAGCCAGTCTCTCCCTGGGCTCTGGATTCTGGCATGCAACAGG
481    TAGTGCAGGGGACTTTTCTCGGTCAGAGAGGGACCCGAGACCTAAGACCGTACGTTGTCC  540

179    R  H  S  S  R  R  L  L  R  A  I  P  R  Q  V  A  Q  T  L  Q  198
       AAGACATTCAAGTCGGCGATTGCTGAGAGCCATTCCTCGCCAGGTCGCCCAGACACTGCA
541    TTCTGTAAGTTCAGCCGCTAACGACTCTCGGTAAGGAGCGGTCCAGCGGGTCTGTGACGT  600

199    A  D  V  L  W  Q  M  G  Y  T  G  A  N  V  R  V  A  V  F  D  218
       GGCAGATGTGCTGTGGCAGATGGGATACACAGGTGCTAATGTCAGAGTTGCTGTTTTTGA
601    CCGTCTACACGACACCGTCTACCCTATGTGTCCACGATTACAGTCTCAACGACAAAAACT  660

219    T  G  L  S  E  K  H  P  H  F  K  N  V  K  E  R  T  N  W  T  238
       TACTGGGCTCAGTGAGAAGCATCCGCATTTTAAGAATGTGAAGGAGAGAACCAACTGGAC
661    ATGACCCGAGTCACTCTTCGTAGGCGTAAAATTCTTACACTTCCTCTCTTGGTTGACCTG  720
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239   N  E  R  T  L  D  D  G  L  G  H  G  T  F  V  A  G  V  I  A   258
      CAATGAGCGGACCCTGGATGATGGGCTAGGCCATGGCACATTCGTTGCAGGTGTGATTGC
721   GTTACTCGCCTGGGACCTACTACCCGATCCGGTACCGTGTAAGCAACGTCCACACTAACG   780

259   S  M  R  E  C  Q  G  F  A  P  D  A  E  L  H  I  F  R  V  F   278
      CAGCATGAGGGAGTGCCAAGGATTTGCTCCAGATGCAGAGCTGCACATCTTCAGGGTCTT
781   GTCGTACTCCCTCACGGTTCCTAAACGAGGTCTACGTCTCGACGTGTAGAAGTCCCAGAA   840

279   T  N  N  Q  V  S  Y  T  S  W  F  L  D  A  F  N  Y  A  I  L   298
      TACCAACAATCAGGTGTCTTACACATCTTGGTTTCTGGATGCCTTCAACTATGCCATCCT
841   ATGGTTGTTAGTCCACAGAATGTGTAGAACCAAAGACCTACGGAAGTTGATACGGTAGGA   900

299   K  K  M  D  V  L  N  L  S  I  G  G  P  D  F  M  D  H  P  F   318
      AAAGAAGATGGACGTTCTCAACCTTAGCATCGGTGGGCCCGACTTCATGGATCATCCGTT
901   TTTCTTCTACCTGCAAGAGTTGGAATCGTAGCCACCCGGGCTGAAGTACCTAGTAGGCAA   960

319   V  D  K  V  W  E  L  T  A  N  N  V  I  M  V  S  A  I  G  N   338
      TGTTGACAAGGTGTGGGAATTAACAGCTAACAATGTAATTATGGTTTCTGCTATTGGCAA
961   ACAACTGTTCCACACCCTTAATTGTCGATTGTTACATTAATACCAAAGACGATAACCGTT  1020

339   D  G  P  L  Y  G  T  L  N  N  P  A  D  Q  M  D  V  I  G  V   358
      TGATGGACCTCTCTATGGCACTCTGAATAACCCTGCTGATCAGATGGATGTGATTGGAGT
1021  ACTACCTGGAGAGATACCGTGAGACTTATTGGGACGACTAGTCTACCTACACTAACCTCA  1080

359   G  G  I  D  F  E  D  N  I  A  R  F  S  S  R  G  M  T  T  W   378
      GGGTGGCATTGACTTTGAAGATAACATCGCTCGCTTTTCTTCCAGGGGAATGACTACCTG
1081  CCCACCGTAACTGAAACTTCTATTGTAGCGAGCGAAAAGAAGGTCCCCTTACTGATGGAC  1140

379   E  L  P  G  G  Y  G  R  V  K  P  D  I  V  T  Y  G  A  G  V   398
      GGAATTACCAGGAGGCTATGGTCGTGTGAAGCCTGACATTGTCACCTATGGTGCTGGAGT
1141  CCTTAATGGTCCTCCGATACCAGCACACTTCGGACTGTAACAGTGGATACCACGACCTCA  1200

399   R  G  S  G  V  K  G  G  C  R  A  L  S  G  T  S  V  A  S  P   418
      GCGGGGTTCCGGTGTGAAAGGGGGCTGCCGTGCACTCTCAGGGACCAGTGTCGCTTCCCC
1201  CGCCCCAAGGCCACACTTTCCCCCGACGGCACGTGAGAGTCCCTGGTCACAGCGAAGGGG  1260

419   V  V  A  G  A  V  T  L  L  V  S  T  V  Q  K  R  E  L  V  N   438
      AGTGGTCGCTGGGGCCGTCACCTTGTTAGTAAGCACAGTACAGAAGCGGGAGCTGGTGAA
1261  TCACCAGCGACCCCGGCAGTGGAACAATCATTCGTGTCATGTCTTCGCCCTCGACCACTT  1320

439   P  A  S  V  K  Q  A  L  I  A  S  A  R  R  L  P  G  V  N  M   458
      TCCTGCCAGTGTGAAGCAAGCTTTGATAGCGTCAGCCCGGAGACTTCCTGGGGTCAACAT
1321  AGGACGGTCACACTTCGTTCGAAACTATCGCAGTCGGGCCTCTGAAGGACCCCAGTTGTA  1380

459   F  E  Q  G  H  G  K  L  D  L  L  R  A  Y  Q  I  L  S  S  Y   478
      GTTCGAGCAAGGTCATGGCAAGTTGGATCTGCTGCGAGCTTATCAGATCCTCAGCAGCTA
1381  CAAGCTCGTTCCAGTACCGTTCAACCTAGACGACGCTCGAATAGTCTAGGAGTCGTCGAT  1440
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 479   K  P  Q  A  S  L  S  P  S  Y  I  D  L  T  E  C  P  Y  M  W    498
       TAAACCGCAGGCAAGCCTGAGTCCTAGCTACATCGACCTGACTGAGTGTCCCTACATGTG
1441   ATTTGGCGTCCGTTCGGACTCAGGATCGATGTAGCTGGACTGACTCACAGGGATGTACAC   1500

 499   P  Y  C  S  Q  P  I  Y  Y  G  G  M  P  T  I  V  N  V  T  I    518
       GCCCTACTGCTCCCAGCCTATCTACTATGGAGGAATGCCAACAATCGTTAATGTCACCAT
1501   CGGGATGACGAGGGTCGGATAGATGATACCTCCTTACGGTTGTTAGCAATTACAGTGGTA   1560

 519   L  N  G  M  G  V  T  G  R  I  V  D  K  P  E  W  R  P  Y  L    538
       CCTCAATGGCATGGGCGTCACAGGAAGAATTGTGGATAAGCCTGAGTGGCGACCCTATTT
1561   GGAGTTACCGTACCCGCAGTGTCCTTCTTAACACCTATTCGGACTCACCGCTGGGATAAA   1620

 539   P  Q  N  G  D  N  I  E  V  A  F  S  Y  S  S  V  L  W  P  W    558
       ACCACAGAATGGAGACAACATTGAAGTGGCCTTCTCCTACTCCTCAGTGTTGTGGCCCTG
1621   TGGTGTCTTACCTCTGTTGTAACTTCACCGGAAGAGGATGAGGAGTCACAACACCGGGAC   1680

 559   S  G  Y  L  A  I  S  I  S  V  T  K  K  A  A  S  W  E  G  I    578
       GTCAGGTTACCTTGCCATCTCCATTTCTGTGACCAAGAAGGCAGCTTCCTGGGAAGGCAT
1681   CAGTCCAATGGAACGGTAGAGGTAAAGACACTGGTTCTTCCGTCGAAGGACCCTTCCGTA   1740

 579   A  Q  G  H  I  M  I  T  V  A  S  P  A  E  T  E  L  H  S  G    598
       CGCTCAGGGCCACATCATGATCACAGTGGCGTCCCCAGCAGAGACAGAGTTACACAGTGG
1741   GCGAGTCCCGGTGTAGTACTAGTGTCACCGCAGGGGTCGTCTCTGTCTCAATGTGTCACC   1800

 599   A  E  H  T  S  T  V  K  L  P  I  K  V  K  I  I  P  T  P  P    618
       TGCGGAGCACACTTCCACCGTGAAGCTGCCCATCAAGGTGAAGATCATTCCCACCCCTCC
1801   ACGCCTCGTGTGAAGGTGGCACTTCGACGGGTAGTTCCACTTCTAGTAAGGGTGGGGAGG   1860

 619   R  S  K  R  V  L  W  D  Q  Y  H  N  L  R  Y  P  P  G  Y  F    638
       TCGGAGCAAGAGAGTCCTCTGGGACCAGTACCACAACCTCCGCTACCCACCTGGCTACTT
1861   AGCCTCGTTCTCTCAGGAGACCCTGGTCATGGTGTTGGAGGCGATGGGTGGACCGATGAA   1920

 639   P  R  D  N  L  R  M  K  N  D  P  L  D  W  N  G  D  H  V  H    658
       CCCCAGGGACAACTTGCGGATGAAGAATGACCCTTTAGACTGGAATGGCGACCACGTCCA
1921   GGGGTCCCTGTTGAACGCCTACTTCTTACTGGGAAATCTGACCTTACCGCTGGTGCAGGT   1980

 659   T  N  F  R  D  M  Y  Q  H  L  R  S  M  G  Y  F  V  E  V  L    678
       CACCAACTTCAGGGACATGTACCAGCATCTGCGCAGCATGGGCTACTTCGTGGAGGTGCT
1981   GTGGTTGAAGTCCCTGTACATGGTCGTAGACGCGTCGTACCCGATGAAGCACCTCCACGA   2040

 679   G  A  P  F  T  C  F  D  A  T  Q  Y  G  T  L  L  L  V  D  S    698
       CGGCGCCCCATTCACATGTTTTGACGCCACACAGTATGGCACTTTGCTGCTGGTGGACAG
2041   GCCGCGGGGTAAGTGTACAAAACTGCGGTGTGTCATACCGTGAAACGACGACCACCTGTC   2100

 699   E  E  E  Y  F  P  E  E  I  A  K  L  R  R  D  V  D  N  G  L    718
       TGAGGAAGAGTACTTCCCTGAGGAGATTGCTAAGCTGAGGAGGGATGTGGACAATGGCCT
2101   ACTCCTTCTCATGAAGGGACTCCTCTAACGATTCGACTCCTCCCTACACCTGTTACCGGA   2160

 719   S  L  V  I  F  S  D  W  Y  N  T  S  V  M  R  K  V  K  F  Y    738
       TTCCCTCGTCATCTTCAGTGACTGGTACAACACTTCTGTTATGAGAAAAGTGAAGTTTTA
2161   AAGGGAGCAGTAGAAGTCACTGACCATGTTGTGAAGACAATACTCTTTTCACTTCAAAAT   2220
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SUBSTITUTE SHEET (RULE 26)

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 739   D  E  N  T  R  Q  W  W  M  P  D  T  G  G  A  N  I  P  A  L    758
       TGATGAAAACACCAGGCAGTGGTGGATGCCAGACACCGGAGGAGCGAACATCCCAGCTCT
2221   ACTACTTTTGTGGTCCGTCACCACCTACGGTCTGTGGCCTCCTCGCTTGTAGGGTCGAGA   2280

 759   N  E  L  L  S  V  W  N  M  G  F  S  D  G  L  Y  E  G  E  F    778
       GAATGAGCTGCTGTCTGTGTGGAACATGGGGTTCAGTGACGGCCTATATGAAGGGGAGTT
2281   CTTACTCGACGACAGACACACCTTGTACCCCAAGTCACTGCCGGATATACTTCCCCTCAA   2340

 779   V  L  A  N  H  D  M  Y  Y  A  S  G  C  S  I  A  K  F  P  E    798
       TGTCCTGGCAAACCATGACATGTACTATGCGTCGGGGTGCAGCATCGCCAAGTTTCCAGA
2341   ACAGGACCGTTTGGTACTGTACATGATACGCAGCCCCACGTCGTAGCGGTTCAAAGGTCT   2400

 799   D  G  V  V  I  T  Q  T  F  K  D  Q  G  L  E  V  L  K  Q  E    818
       AGATGGCGTCGTGATCACACAGACTTTCAAGGACCAAGGATTGGAGGTCTTAAAACAAGA
2401   TCTACCGCAGCACTAGTGTGTCTGAAAGTTCCTGGTTCCTAACCTCCAGAATTTTGTTCT   2460

 819   T  A  V  V  E  N  V  P  I  L  G  L  Y  Q  I  P  S  E  G  G    838
       GACAGCAGTTGTGGAAAATGTTCCCATTTTGGGGCTTTATCAGATTCCATCTGAAGGTGG
2461   CTGTCGTCAACACCTTTTACAAGGGTAAAACCCCGAAATAGTCTAAGGTAGACTTCCACC   2520

 839   G  R  I  V  L  Y  G  D  S  N  C  L  D  D  S  H  R  Q  K  D    858
       AGGCCGGATCGTGCTGTATGGAGACTCCAACTGCTTGGATGACAGTCACAGACAGAAGGA
2521   TCCGGCCTAGCACGACATACCTCTGAGGTTGACGAACCTACTGTCAGTGTCTGTCTTCCT   2580

 859   C  F  W  L  L  D  A  L  L  Q  Y  T  S  Y  G  V  T  P  P  S    878
       CTGCTTTTGGCTTCTGGATGCGCTCCTTCAGTACACATCCTATGGCGTGACCCCTCCCAG
2581   GACGAAAACCGAAGACCTACGCGAGGAAGTCATGTGTAGGATACCGCACTGGGGAGGGTC   2640

 879   L  S  H  S  G  N  R  Q  R  P  P  S  G  A  G  L  A  P  P  E    898
       CCTCAGCCATTCAGGGAACCGGCAGCGCCCACCTAGCGGAGCCGGCTTGGCCCCTCCTGA
2641   GGAGTCGGTAAGTCCCTTGGCCGTCGCGGGTGGATCGCCTCGGCCGAACCGGGGAGGACT   2700

 899   R  M  E  G  N  H  L  H  R  Y  S  K  V  L  E  A  H  L  G  D    918
       AAGGATGGAAGGAAACCACCTCCATCGGTACTCCAAAGTTCTTGAAGCCCACTTGGGAGA
2701   TTCCTACCTTCCTTTGGTGGAGGTAGCCATGAGGTTTCAAGAACTTCGGGTGAACCCTCT   2760

 919   P  K  P  R  P  L  P  A  C  P  H  L  S  W  A  K  P  Q  P  L    938
       CCCGAAACCTCGGCCCCTGCCAGCCTGTCCACATTTGTCATGGGCCAAGCCACAGCCTTT
2761   GGGCTTTGGAGCCGGGGACGGTCGGACAGGTGTAAACAGTACCCGGTTCGGTGTCGGAAA   2820

 939   N  E  T  A  P  S  N  L  W  K  H  Q  K  L  L  S  I  D  L  D    958
       GAATGAGACGGCACCCAGTAATCTTTGGAAACATCAGAAGCTGCTCTCCATTGACCTGGA
2821   CTTACTCTGCCGTGGGTCATTAGAAACCTTTGTAGTCTTCGACGAGAGGTAACTGGACCT   2880

 959   K  V  V  L  P  N  F  R  S  N  R  P  Q  V  R  P  L  S  P  G    978
       CAAAGTAGTGTTACCCAACTTTCGATCCAATCGCCCTCAAGTGAGACCTTTGTCCCCTGG
2881   GTTTCATCACAATGGGTTGAAAGCTAGGTTAGCGGGAGTTCACTCTGGAAACAGGGGACC   2940
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 979   E  S  G  A  W  D  I  P  G  G  I  M  P  G  R  Y  N  Q  E  V    998
       AGAGAGTGGTGCCTGGGACATTCCTGGAGGGATCATGCCTGGCCGCTACAACCAGGAGGT
2941   TCTCTCACCACGGACCCTGTAAGGACCTCCCTAGTACGGACCGGCGATGTTGGTCCTCCA   3000

 999   G  Q  T  I  P  V  F  A  F  L  G  A  M  V  A  L  A  F  F  V    1018
       GGGACAGACCATCCCCGTCTTCGCCTTCCTCGGAGCCATGGTGGCCCTGGCCTTCTTTGT
3001   CCCTGTCTGGTAGGGGCAGAAGCGGAAGGAGCCTCGGTACCACCGGGACCGGAAGAAACA   3060

1019   V  Q  I  S  K  A  K  S  R  P  K  R  R  R  P  R  A  K  R  P    1038
       GGTACAGATCAGCAAGGCCAAGAGCCGGCCGAAGCGGAGGAGGCCCAGGGCAAAGCGTCC
3061   CCATGTCTAGTCGTTCCGGTTCTCGGCCGGCTTCGCCTCCTCCGGGTCCCGTTTCGCAGG   3120

1039   Q  L  A  Q  Q  A  H  P  A  R  T  P  S  V                      1052
       ACAACTTGCACAGCAGGCCCACCCTGCAAGGACCCCATCAGTGTGAGCATCGCAGTAGCC
3121   TGTTGAACGTGTCGTCCGGGTGGGACGTTCCTGGGGTAGTCACACTCGTAGCGTCATCGG   3180

       AGCCACAGAAGCTAACAAGCCTTGAACCACTCTGGTGGCCACACAGCGCCTCAGAGAGCA
3181   TCGGTGTCTTCGATTGTTCGGAACTTGGTGAGACCACCGGTGTGTCGCGGAGTCTCTCGT   3240

       TTCTGGGAAGTGCCTGTTTCCGAGGACCCTGTCTCCAGCTTGTGGCTATCTTACTGTGTT
3241   AAGACCCTTCACGGACAAAGGCTCCTGGGACAGAGGTCGAACACCGATAGAATGACACAA   3300

       CTGCCCAGGCACCTGATGAGGTACATCCTGCAGTGCCTCTCTGTGCTTGGCTCTGGCAGA
3301   GACGGGTCCGTGGACTACTCCATGTAGGACGTCACGGAGAGACACGAACCGAGACCGTCT   3360

       AGGCACCCAGTGACATCAGGCATCAGGCCCAGTGACAGTGCACCAAAGACACAGAGCCTG
3361   TCCGTGGGTCACTGTAGTCCGTAGTCCGGGTCACTGTCACGTGGTTTCTGTGTCTCGGAC   3420

       GAAGGGCTGTCGGGACATACTTTCTACATAACGCTACAACCCTGACCAAGCAAAGACATG
3421   CTTCCCGACAGCCCTGTATGAAAGATGTATTGCGATGTTGGGACTGGTTCGTTTCTGTAC   3480

       CTTGTTACAGGCTATTTTCTATATTTATTGTGGGAGAGTCACTTTAAAGACTGTGCTAGT
3481   GAACAATGTCCGATAAAAGATATAAATAACACCCTCTCAGTGAAATTTCTGACACGATCA   3540

       TGGAAACAGAGCTGTTGCTGTTGTCAGTCGAGTGCAGTTTTCTGCAGCGATGTCATAAGG
3541   ACCTTTGTCTCGACAACGACAACAGTCAGCTCACGTCAAAAGACGTCGCTACAGTATTCC   3600

       AGTCAGATTCCGTGACCTCCTCTTTGATGGAGGACACACTGAACTGAAGGGGACTTGCGC
3601   TCAGTCTAAGGCACTGGAGGAGAAACTACCTCCTGTGTGACTTGACTTCCCCTGAACGCG   3660

       GGATGTGGGAGATGCAAGCCTTCGCTTTATTTTTTTATAACTATCAACTGCCATCATGTT
3661   CCTACACCCTCTACGTTCGGAAGCGAAATAAAAAAATATTGATAGTTGACGGTAGTACAA   3720

       TTGTAATTTGGGGATCTTGATTTCACCGTTGTTGGTGAAGGAAATTTTCAATAAATATGC
3721   AACATTAAACCCCTAGAACTAAAGTGGCAACAACCACTTCCTTTAAAAGTTATTTATACG   3780

       ATAACCTT
3781   TATTGGAA  3788
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